4, Vol. 51, 1985

PIBSBB 51 (4) 391-536 (1985)

ISSN 0073-6600

Biological
Sciences
Part B

PROCEEDINGS OF THE INDIAN NATIONAL SCIENCE ACADEMY

INDIAN NATIONAL SCIENCE ACADEMY
NEW DELHI

Proceedings of the Indian National Scientific Academy Part B

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foreign: $ 50.00
Single Issue
inland: Rs. 30.00
foreign: $ 10.00

Editorial Office
The Indian National Science Academy, Bahadur Shah Zafar Marg, New Delhi 110002, India

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Proceedings of the Indian National Science Academy Part B, is published in alternate months, i.e., February, April, June, August, October and December.

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Purpose and Scope

Part B of the Proceedings is devoted to papers embodying results of original research in any branch of Life Sciences. The major emphasis of the journal will be on publication of studies especially those with Inter-disciplinary implications relating to Physiology, Cell Biology, Cytogenetics, Genetics, Ecology, Biochemistry, Microbiology, Pathology, Endocrinology, Physiology of Reproduction, etc., relating to plants and animals.

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Proc. Indian natn. Sci. Acad. B51 No 4 pp 391-404 (1986)

Critical Study of the Gill Area of a Free-swimming River Carp, *Catla catla* and an Air-breathing Perch, *Anabas testudineus*

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(Received 3 May 1985)

Measurements of gill dimensions of the climbing perch, *Anabas testudineus* and a riverine carp, *Catla catla* have been made to compare different sampling techniques.

The gills in *Anabas* are far more heterogeneous than those of *Catla*. *Anabas* has a small number (610) of shorter (2.33mm) filaments whereas *Catla* of the same body weight (100 gm) has a greater number (3792) of longer (5.52 mm) filaments. Consequently, the total filament length of *Catla* (20949 mm) is more than 14 times that of *Anabas* (1422 mm). The secondary lamellar frequency in the free-swimming *Catla* was higher (27/mm on each side of a filament) than in *Anabas* (21/mm), which also has accessory air-breathing organs. The area of an average secondary lamella in *Anabas* (0.1093 mm^2), however, was more than 4 times that of *Catla* (0.0251 mm^2). As a consequence of these differences in the component parameters the total gill area of *Catla* (28536.3 mm^2) is nearly 4.5 times greater than that of *Anabas* (6483.3 mm^2).

The distribution of area between different gill arches is more variable in *Anabas* than *Catla*, but in both species if only one arch is sampled, the second gives the least error.

It is concluded that in air-breathing species where the gills are generally heterogeneous a wide sample is necessary for gill measurements, whereas in fishes with more homogeneous gills a much smaller sample from a single arch will introduce relatively small errors so long as care is taken to determine which arch is the most representative.

Key Words: Gill area, Carp, Air breathing perch, *Catla*, *Anabas*

Introduction

During the past thirty years a number of studies have been made of the surface area of the gills of Indian fishes especially those which breathe air (Dubale 1951, Saxena

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1962, Hughes et al. 1973, Hughes et al. 1974, Ojha & Munshi 1974, Munshi 1976, Munshi et al. 1980). These studies have extended our knowledge and have confirmed the generally held view (Carter 1957) that the gills of air-breathing species are less well developed than those which breathe entirely in water. Furthermore, the studies have often included measurements of the surface area of the air-breathing organs and it has also been established that although these may not necessarily have a greater surface area than the gills, nevertheless they are better adapted to a gas exchange function by virtue of the thinner barrier separating the respiratory medium from the blood. Combined studies of surface area and thickness of this barrier have established a number of values for the diffusing capacity of these gas exchange organs which provide a better guide to their overall function. Thus the air-breathing organs of *Anabas* have a tissue barrier of less than 1 μm whereas in the secondary lamellae of the gills it is often greater than 10 μm (Hughes & Munshi 1968). This greater thickness does not form such a serious barrier to the outward diffusion of carbon dioxide which has also been shown to be a very important function for the gills in these bimodal breathers (Hughes 1966a, Singh 1976). Unfortunately, there has been little standardisation in methods used for measurement of thickness of the water/blood barrier (Hughes 1982, 1984b).

The technique for measurement of surface areas of respiratory structures has developed during this period but in all cases depends upon sampling procedures as it is impossible to measure the total surface directly, except perhaps in the case of the skin. Improvements in sampling technique have included the method of weighting introduced by Muir and Hughes (1969) which estimates the surface area and spacing of secondary lamellae according to their relative numbers in the regions that have been sampled. It has also become customary to pay special attention to the dimensions of the secondary lamellae on the second gill arch as this is fairly representative in water-breathing species where the methods were established. This procedure has a number of disadvantages when applied to air-breathing fishes where the degree of development of different gill arches is much more varied. Thus detailed measurements using all the gill arches have been reported. Furthermore, controlled measurements have been made during the last 10 years using water-breathing species and these have led to some modifications (Hughes 1982, 1984a, b) in the normal procedure initially established by Muir and Hughes (1969) and Hughes and Morgan (1973).

The present study was, therefore, undertaken in order to make detailed comparisons of the morphometry of the gills of individual specimens of *Catla catla* and *Anabas testudineus* of approximately the same body weight.

The results give rise to a number of suggestions regarding the types of procedure which should be carried out in future measurements. They also indicate the range of errors which might arise as a result of the size of the samples taken for determination of secondary lamellar frequency and area. As a result of this work it is also possible to suggest the minimum sample size which can be taken while keeping the error fairly small.

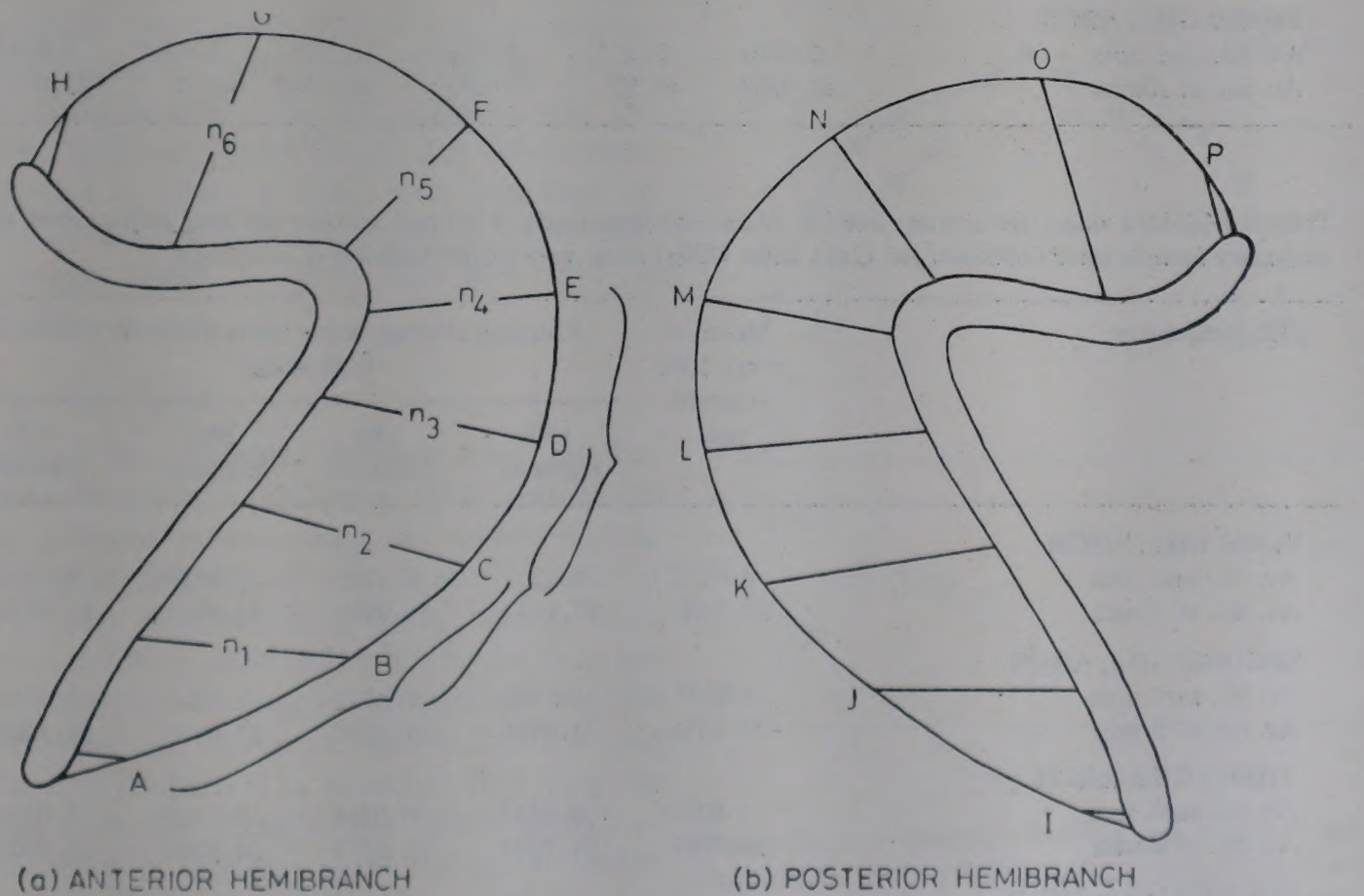
Materials and Methods

Catla catla was collected from the River Ganges and *Anabas testudineus* from swamps near Katihar, India. These fishes were kept in the Post-Graduate Department of Zoology, Bhagalpur University before specimens of about 100g were killed and their heads fixed in aqueous Bouin's solution and stored at 4°C to minimise shrinkage. The

individual gill arches from both sides were separated and used for measurement of filament length, frequency of secondary lamellae and secondary lamellar area as in previous studies (Muir & Hughes 1969, Hughes & Morgan 1973).

In this case measurements were made using every tenth (*Catla*) or fifth (*Anabas*) filament along both hemibranchs of each of all gill arches on right side of the fish (figures 1a, 1b). For the estimate of total filament length measurements also included the first and last filaments. In addition measurements were made of the frequency and bilateral surface area of samples from base, middle and tip of the measured filaments. Running averages of secondary lamellar measurements were made for the estimation of total gill area. Calculations of surface area utilised the standard method of weighting (Muir & Hughes 1969, Hughes & Morgan 1973). This involved a considerable increase in the normal amount of measurement for individual specimens and it was for this reason that it was only applied to a single specimen of each species. As the whole procedure of gill area measurement is very time consuming, it is advisable to have some information concerning the relative errors introduced if the number of samples is reduced. Such procedures have become quite common for water-breathing species where only the second or third arch on one side is normally sampled and the secondary lamellar area and frequency is measured for about 6–10 filaments on these arches.

These analyses were greatly assisted by the use of a Hewlett Packard 9810 Calculator and Apple II microcomputer.



Figures 1a & b Diagrammatic representations of anterior (1a) and posterior hemibranchs (1b) to show the sampled regions for filament length (A–P) and lamellar frequency and area (n1–n6). Sampled regions for running averages of component parameters are also indicated by brackets

Results

Tables 1 and 2 summarise the results on lamellar frequency and area of *Anabas* and *Catla* using various combinations of samplings and table 3 indicates an overall comparison between the two specimens. It should be noted that in the case of *Anabas* there are only three pairs of gill arches which have functional gill filaments and the third of these has a relatively small number of filaments. In fact the total number of filaments in this specimen was only 610,

Table 1 *Weighted values for average number of sec. lam/mm (both sides=n) and average bilateral surface area of a secondary lamella (mm^2=bl) obtained for* Anabas testudineus *(Bloch) using various combinations of samplings*

Gill parameters	Mean of every 5th adjacent pair	Mean of every 10th adjacent pair	Running average using measurements obtained from every			
			5th fil.	10th fil.	15th fil.	20th fil.
FIRST GILL ARCH						
Av. bil. surf. area	0.1219	0.1163	0.1221	0.1136	0.1152	0.0982
Av. no. of sl/mm	41.0344	41.7994	41.0106	41.4561	40.6697	40.6545
SECOND GILL ARCH						
Av. bil. surf. area	0.1034	0.1012	0.1032	0.0987	0.1088	0.0913
Av. no. of sl/mm	41.8222	40.7565	41.7780	40.6061	41.6287	39.7773
THIRD GILL ARCH						
Av. bil. surf. area	0.0701	0.0735	0.0705	0.0732	0.0707	0.0688
Av. no. of sl/mm	44.9839	44.50	45.0732	44.6667	46.0	45.0

Table 2 *Weighted values for average number of sec. lam/mm (both sides) and average bilateral surface area of a secondary lamella (mm^2) obtained for* Catla catla *(100g) using various combination of samplings*

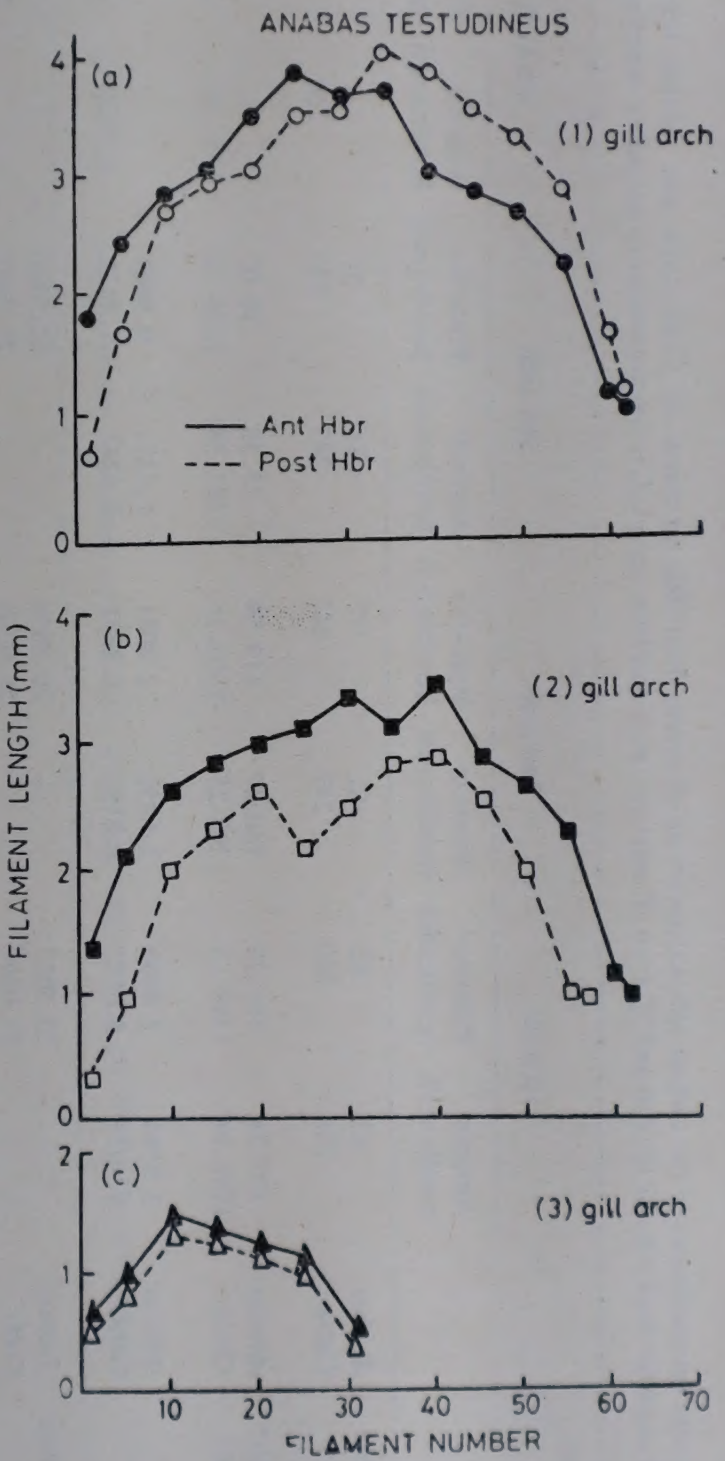
Gill parameters	Mean of every 10th adjacent pair	Running average using measurements obtained from every			
		10th filament	20th filament	30th filament	40th filament
FIRST GILL ARCH					
Av. bil. surf. area	0.0252	0.0253	0.0259	0.0257	0.0259
Av. no. of sl/mm	52.1609	52.1197	51.9988	53.0114	53.4501
SECOND GILL ARCH					
Av. bil. surf. area	0.0247	0.0248	0.0247	0.0233	0.0243
Av. no. of sl/mm	53.8558	53.8496	53.6236	53.9973	54.3809
THIRD GILL ARCH					
Av. bil. surf. area	0.0260	0.0257	0.0264	0.0268	0.0246
Av. no. of sl/mm	54.7088	54.7270	55.0573	54.9763	55.0007
FOURTH GILL ARCH					
Av. bil. surf. area	0.0250	0.0247	0.0241	0.0233	0.0234
Av. no. of sl/mm	56.4470	56.0978	56.0471	56.1463	57.3292

whereas the specimen of *Catla* of the same body weight (100g) had 3792 filaments. Consequently, the total filament length in *Catla* (20949.4 mm) is more than 14 times that of the *Anabas* specimen (1422.1 mm). The frequency of the secondary lamellae was higher in the more actively swimming species (54/mm for both sides of the filament (n) as against 42/mm). However, the area of an average secondary lamella from the climbing perch specimen (0.1093 mm^2) was more than 4 times that of *Catla* (0.0251 mm^2). As a consequence of these differences in the component measurements, total gill area of *Catla* (28536.3 mm^2) is about 4.5 times greater than that of *Anabas* (6483.3 mm^2).

A detailed analysis of the distribution of different gill parameters within the gill arches is shown in figures 2–8. From figures 2a, 2b, 3a, 3b it is apparent that the number of filaments on the first two arches of *Anabas* is about one quarter that on the corresponding arches of *Catla* and their average length in *Catla* is about 2 times greater. On the third arch of *Anabas* (figure 2c) the filament number is about 8 times less than that on the same arch of *Catla* (figure 3c) and their average length is about one fifth that of *Catla*.

Filament Length

From the detailed measurements of filament length it is possible to plot their length at different points along the arches and to compare the relative length of filaments of the two hemibranchs. Such studies have been made in other fishes but so far they are not available for any Indian species. Results obtained are shown in figures 2a, b, c and 3a, b, c, d where it is apparent that there are not only differences in the relative length of filaments at different points along the arches but also that this relationship varies in different arches. It should be noted that in these



Figures 2a, b, c Diagrams to show the length of gill filaments on the two hemibranchs of each of the three gill arches of *Anabas*. It should be noted that the filaments are numbered from ventral to dorsal

Table 3 *Detailed measurements of the various gill-parameters on all arches from 100g specimens of* Catla catla *and* Anabas testudineus. *Values for expected total areas for both sides of the fish and % error if only one arch had been sampled for measurements of secondary lamellar frequency and area*

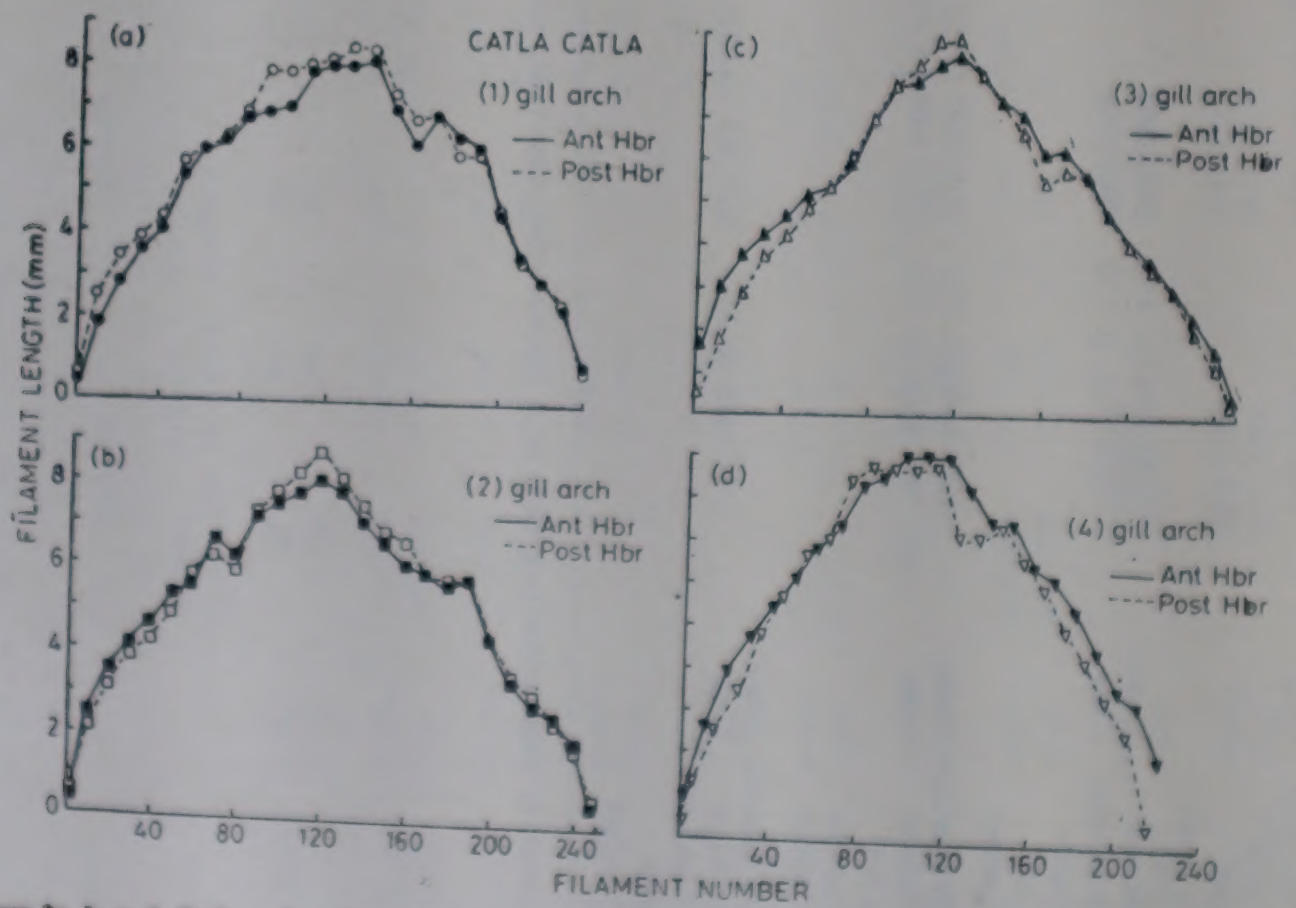
Gill parameters		1st arch		2nd arch		3rd arch		4th arch		Totals or means
		Anterior hemibranch	Posterior hemibranch	Anterior hemibranch	Posterior hemibranch	Anterior hemibranch	Posterior hemibranch	Anterior hemibranch	Posterior hemibranch	Two sides of fish
No. of filaments	*Anabas*	62	62	62	57	31	31	—	—	610
	Catla	238	240	247	247	247	243	220	214	3792
Total fil. length* (mm)	*Anabas*	177.28	182.28	166.47	119.59	35.38	30.03	—	—	1422.1
	Catla	1286.94	1348.13	1293.25	1306.16	1342.38	1286.16	1355.63	1256.06	20949.4
Av. fil. length (mm)	*Anabas*	2.8594	2.9400	2.6850	2.0981	1.1411	0.9688	—	—	2.3313
	Catla	5.4073	5.6172	5.2358	5.2881	5.4347	5.2928	6.1620	5.8694	5.5246
Sec. lamellae/mm (one side)	*Anabas*	—	20.5053	—	20.8890	—	22.5366	—	—	20.8459
	Catla		26.0599		26.9248		27.3635		28.0978	27.1098
Av. bil. sec. lamellar area (mm^2)	*Anabas*	—	0.12210	—	0.1032	—	0.0705	—	—	0.1093
	Catla		0.0253		0.0248		0.0257		0.0247	0.0251
Total no. of sec. lamellae	*Anabas*	7270.35	7475.41	6954.78	4996.23	1594.68	1353.54	—	—	59290.04
	Catla	67075.05	70264.26	69640.99	70336.19	73464.43	70387.67	76180.44	70585.04	1135868.1
Gill area/mm Fil. (mm^2)	*Anabas*	5.0074	5.0074	4.3115	4.3115	3.1777	3.1777	—	—	4.55898
	Catla	1.3186	1.3186	1.3385	1.3385	1.4065	1.4065	1.3880	1.3880	1.36216
Total gill area/ hemibranch (mm^2)	*Anabas*	887.7119	912.7489	717.7354	515.612	112.4270	95.4263	—	—	6483.3236
	Catla	1696.9590	1777.6442	1727.1353	1744.376	1888.0574	1808.9840	1881.6100	1743.4100	28536.3520
Total gill area/ arch (mm^2)	*Anabas*	1800.4608		1233.3477		207.8533		—		6483.3236
	Catla	3474.6032		3471.5119		3697.0414		3625.02		28536.3520
Total gill area/ arch both sides of fish	*Anabas*	3600.92166		2466.6954		415.7066		—		6483.3236
	Catla	6949.2064		6943.0238		7394.0828		7250.04		28530.3520

Total gill area/ arch both sides if 1st arch sampled (& % error)	*Anabas*	3600.9216	2864.8337 (16%)	655.0681 (57.6%)	—	7120.8234 (9.83%)
	Catla	6949.2064	6858.1640 (1.3%)	6931.9856 (6.2%)	6887.5488 (5%)	27623.9040 (3.2%)
Total gill area/ arch both sides if 2nd arch sampled (& % error)	*Anabas*	3100.4859 (13.9%)	2466.6954	664.0304 (35.7%)	—	6131.2117 (5.4%)
	Catla	7038.2718 (1.3%)	6943.0238	7020.8302 (5.04%)	6975.8238 (3.8%)	27977.9480 (1.96%)
Total gill area/arch both sides of fish if 3rd arch sampled (& % error)	*Anabas*	2285.1476 (36.5%)	1818.0257 (26%)	415.7066	—	4518.88 (30.2%)
	Catla	7412.4518 (6.7%)	7312.1402 (5.3%)	7394.0828	7346.6838	29465.3570 (3.3%)
Total gill area/arch both sides of fish if 4th arch sampled (& % error)	*Anabas*	—	—	—	—	—
	Catla	7314.9542 (5.3%)	7215.962 (3.9%)	7296.8270 (1.3%)	7250.04 (1.3%)	29107.783 (2%)

*If measured by older method (not using 1st & last filaments) these values would have been about 4% less

figures filament No. 1 is at the ventral end of the arch whereas in previous studies (e.g. Hughes 1972, Adeney & Hughes 1977, Morgan 1971) filament 1 has been the most dorsal. This form of presentation also emphasises a well-known feature of the gills of *Anabas*, where except for the first gill arch the filaments of anterior hemibranchs are significantly longer than corresponding filaments of the posterior hemibranch. Such differences in the filament lengths of the two hemibranchs of each arch help to produce gill sieve suitable for swampy waters.

The more uniform development of the gills in a water breathing species, such as *Catla* is evident and the far larger number of gill filaments and their greater length is also apparent (figures 3a–3d). The corresponding filaments at different points along the four arches are not significantly different. From this plot it can be seen that of the 478 filaments borne on the first gill arch, the length of the ventralmost 170 filaments (numbered from the ventral end of the arch) of the posterior hemibranch is greater than their counterparts on the anterior hemibranch. For the next 20 filaments the anterior filaments are longer than the posterior and the most dorsal filaments of both hemibranchs are approximately the same length. Similar differences are found in the other arches with variations in precise details. In general the number of filaments for which the filaments of anterior hemibranch are longer than those of the posterior hemibranch decreases successively from the first to the second, third and fourth arches. In the fourth gill arch almost all filaments of the anterior hemibranch have a greater length than their counterpart of the posterior hemibranch. Furthermore, at the



Figures 3a, b, c, d *Catla catla*. Plots to show the length of gill filaments on the two hemibranchs of each of the four gill arches. Note that the filaments are numbered from ventral to dorsal

ventral end of the fourth arch the first filament of the posterior hemibranch is situated opposite the sixth filament of the anterior hemibranch.

Secondary Lamellar Frequency

Lamellar frequency may be expressed as the reciprocal of the distance (d) for each respiratory unit or as the number (n) for each mm on both sides of a filament i.e., $2/d = n$. The former gives a better indication of the tightness of the sieve and the latter is directly used in calculations of total gill area.

The gill filaments of *Catla* are characterised by a higher secondary lamellar frequency (n = 54.2/mm) than in *Anabas* (41.7/mm). Except for the first sample the secondary lamellar frequency in both fishes is almost constant for all gill filaments sampled from different parts of a given hemibranch (figure 4).

Secondary Lamellar Area

Bilateral surface area (bl) of an average secondary lamella showed different trends for the two specimens (figure 4). In *Catla* the average lamellar area is almost equal for samples taken from gill filaments in different parts of a hemibranch. However, in *Anabas* a higher secondary lamellar area was recorded in ventral gill filaments which showed a gradual decrease towards dorsal gill filaments of the sampled hemibranch (figure 4). In both fishes heterogeneity was observed

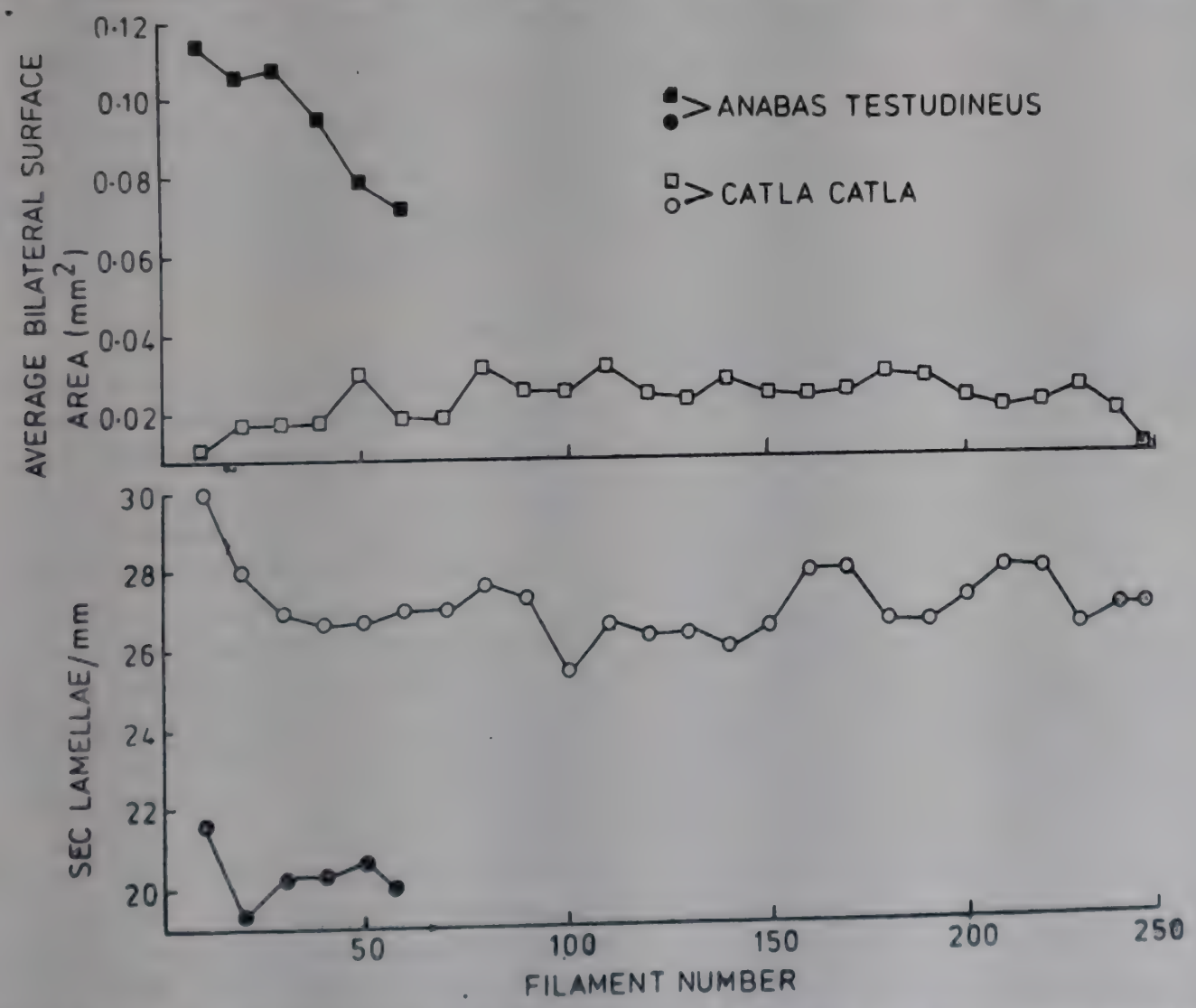


Figure 4 Plots of lamellar frequency and area of single lamella of *Anabas* and *Catla* sampled from various points of a single hemibranch

in the bilateral surface area of secondary lamellae obtained from the base, middle and tip of individual filaments sampled from different regions of their respective hemibranchs (figures 5, 6).

Regional Variations in Gill Area

Variations in total lamellar area for a given number of filaments were also observed in different parts of the hemibranchs of the two fishes. In most hemibrahchs the gill area

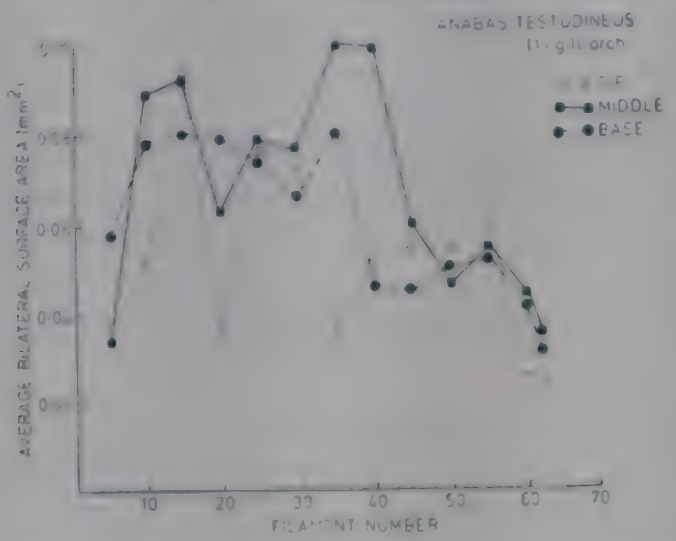


Figure 5 Average bilateral surface area of secondary lamellae sampled from base, middle and tip of the gill filament obtained from 1st gill arch of *Anabas*

Figure 6 Bilateral surface area of a secondary lamella of *Catla* obtained from the base, middle and tip of the various sampled gill filaments from the 1st gill arch

was greatest in the middle region in comparison to their dorsal and ventral parts (figures 7, 8).

Sampling Procedure

For most fish species it has become customary to sample only one arch for the measurement of lamellar frequency and area although it has been appreciated that this may introduce errors (Hughes & Morgan 1973). When this method was applied to the gills of *Anabas* 9.8, 5.4 and 30.2% errors were obtained if the 1st, 2nd or 3rd arches alone were considered to have been sampled respectively. Furthermore, running averages using measurements for every 5th filament gave the best estimate for lamellar frequency and area (tables 1–3). However, the 2nd gill of *Catla* is fairly representative for all the arches as only 1.96% error was introduced when this arch alone was used in the calculations. In *Catla* also the running average of every 10th filament gave the best estimate for average lamellar area and frequency.

Discussion

Anabas testudineus and *Catla catla* inhabit ecologically different habitats. *Anabas* is found in hypoxic and hypercarbic marshy waters whereas *Catla* inhabits normoxic swift running rivers. The respiratory organs of these fishes are suited to the environment in which they live and flourish. *Anabas* has acquired accessory respiratory organs in the form of vascularized channels in the epithelium lining the labyrinthine organs and suprabranchial chambers (Hughes & Munshi 1973) which enable it to survive under adverse ecological conditions. Development of accessory respiratory organs has greatly affected the gill dimensions. The first two arches of *Anabas* bear gill filaments and have most of the gill surface area. The third gill bears a few functional filaments while the fourth has non-functional stumpy filaments.

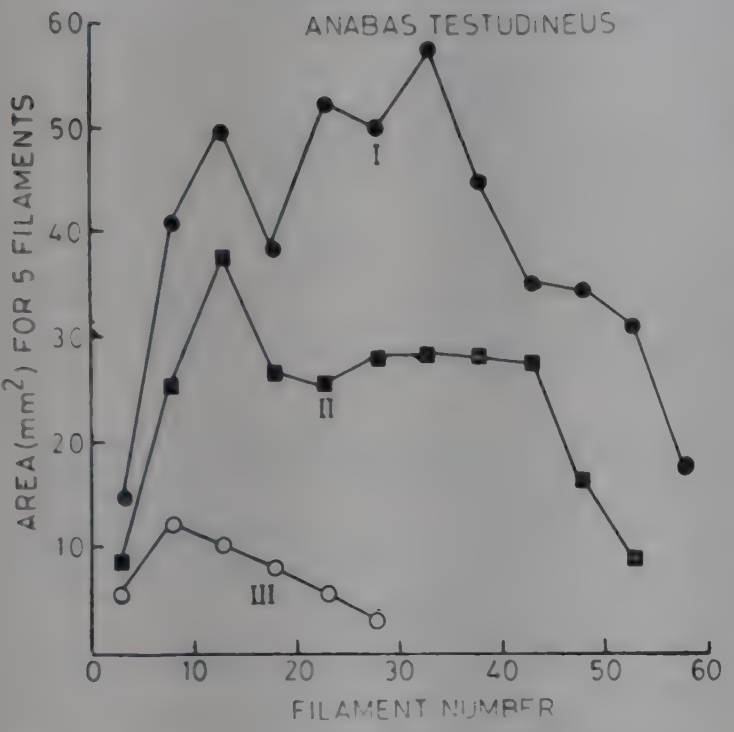


Figure 7 Plots to show the gill area of groups of five filaments sampled at different positions along the three gill arches of *Anabas*

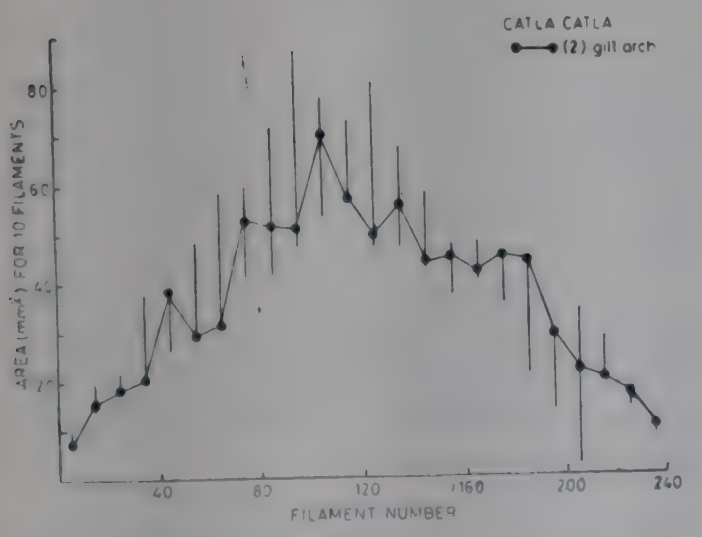


Figure 8 Plots to show the area of groups of five filaments sampled at different positions along the second gill arch of *Catla*. Vertical lines indicate the relative gill area for corresponding filaments of the remaining three arches

Catla catla has more homogeneous gills being an aquatic breather of medium activity and has filaments of similar number and length on each of the arches so that the overall distribution of surface area among the different arches is fairly even. In *Catla* and the first arch of *Anabas* the relative length of filaments of the anterior and posterior hemibranchs of a given arch vary at different points. This variation conforms to the basic geometry of their respective opercular cavities. *Catla* has a more spacious opercular cavity for accommodating a greater number (3792) of large (5.52 mm) gill filaments in comparison to *Anabas* which has to accommodate a smaller number (610) of short (2.33 mm) filaments. This results in fourteen times higher value for total filament length (20949.4 mm) in *Catla* than *Anabas* (1422.1 mm). The small value for *Anabas* is obviously due to the small number of short filaments borne on only three pairs of gill arches. When these values are compared with values (table 4) reported for fishes of varied activities and habitats the relationship can be represented as follows:

Filament length of active marine skipjack tuna (*Katsuwonus pelamis*) > riverine carp (*Catla catla*) > Sluggish toadfish (*Opsanus tau*) > amphibious estuarine goby (*Boleophthalmus boddaerti*) > air-breathing climbing perch (*Anabas testudineus*).

The high filament length of tuna is related to its greater activity in the sea, and lower value for *Anabas* is due to its air-breathing habit in swamps. Secondary lamellar frequency and area are also important parameters which affect total gill area. Lamellar frequency indicates the density of secondary lamellae on gill filaments and thereby reflects the fineness of the sieve. The frequency of secondary lamellae in *Catla* ($1/d' = 27.11$/mm, $n = 54.22$/mm) in about 1.3 times greater than that of *Anabas* ($1/d' = 20.85$/mm). A lower frequency in *Anabas* indicates wider

Table 4 *Summary of the component gill parameters for different fish species of varied activity and habitat*

Gill parameters	*Katsuwonus pelamis* (skipjack tuna) active marine	*Opsanus tau* (toad fish) sluggish marine	*Boleopthalmus boddaerti* (mud skipper) estuarine amphibious goby	*Catla catla* (riverine carp) active freshwater	*Anabas testudineus* (climbing perch) air-breathing swamp
Total filament length (mm)	33498.1	2825.0	2741.6	20949.4	1422.1
Lamellar Frequency (S1/mm)	83.2	22.54	33.54	54.22	41.69
Lamellar Area (mm^2)	2.0940	0.3347	0.0804	0.0251	0.1093
Total gill area (mm^2)	261038.2	21350.0	7364.4	28536.4	6483.32
	Muir & Hughes 1969	Hughes & Gray 1972	Niva et al. 1981	Present work	

inter-lamellar spaces which may be responsible for a greater physiological dead space and thicker boundary layers (Hughes 1966b) in comparison to *Catla* which has much narrower inter-lamellar spaces. These findings indicate surfaces better adapted to aquatic gas exchange in *Catla* in comparison to *Anabas*. When these values are compared with those reported for other fishes from various habitats it is found that skipjack tuna has the highest density of secondary lamellae (1/d′ = 41.61/mm) and the lowest is found in the toadfish (1/d′ = 11.27/mm). Thus relationships for secondary lamellar frequency may be expressed as follows : *Katsuwonus*>*Catla*>*Anabas*>*Boleophthalmus* >*Opsanus*. Lamellar area shows a different trend. Average bilateral surface area in *Anabas* (bl = 0.1093 mm^2) is about four times greater than that of *Catla* (bl = 0.025 mm^2). For hydrodynamic reasons the active fishes have comparatively small lamellae. The diminution in size of secondary lamellae ensures short diffusion distances in the intralamellar water in active fishes. Larger secondary lamellae may also be liable to damage in active fishes living in swift waters of rivers. Thus large numbers of small secondary lamellae on the gill filaments seems to be an adaptation for active fishes. Such finding supports the general conclusion that more active fishes tend to have densely-packed smaller lamellae (Hughes 1966b). Larger secondary lamellae in *Anabas* reduce the resistance to water (and air) flow through them.

Multiplication of total filament length, secondary lamellar frequency and area comprises the total gill surface area of a fish:

$$A = L.n.bl$$

Gill area of *Catla* (2856.4 mm^2) is more than four times greater than that of *Anabas* (6483.3 mm^2). Higher gill area of *Catla* is obviously due to densely packed secondary lamellae on a large number of long gill filaments and enables it to meet the greater oxygen demand. However, in *Anabas* the smaller gill area is supplemented by the air-breathing organs for its total gas exchange in which air breathing dominates (Hughes & Singh 1970).

In absolute terms the value for weight-specific area of *Catla* (285.36 mm^2/gm) is similar to that found for fishes of comparable habit in North America and Europe (Gray 1954, Hughes 1966b).

Suggestions of Sampling Procedures for Future Gill Measurements

Various sampling procedures have been adopted during the last 30 years for measurement of gill area (Gray 1954, Hughes 1966b.

Muir & Hughes 1969, Morgan 1971, Hughes & Gray 1972, Hughes 1982). From the results obtained with *Anabas* it is suggested that in the case of air-breathing and other species having greater heterogeneity in gill structure, it is necessary to take a wider range of samples from all the gill arches. In *Catla catla* which is a purely aquatic breather and has a more homogeneous gill, a much smaller sample will be good enough in morphometric measurements.

Acknowledgements

The authors wish to thank the British Council and Government of Bihar for financial support which enabled Dr Ojha to visit Bristol.

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Proc. Indian natn. Sci. Acad. B51 No. 4 pp 405–412 (1985)

Scanning Electron Microscopic Studies of Wing Microsculpturing in Termites (Isoptera). III. Genera *Odontotermes* and *Macrotermes* (Termitidae, Macrotermitinae)

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Scanning electron microscopic (SEM) studies on wing microsculpturing were made on two species of *Odontotermes* Holmgren and one of *Macrotermes* Holmgren, and the results compared with the earlier light microscope (LM) findings. On both wing surfaces, in addition to the universal papillae, only cuticular rods occur in *Odontotermes* and pimpules in *Macrotermes*. (*i*) *O. obesus* (Rambur): Rods are substraight to sometimes slightly bent angularly or, rarely, S-shaped. The thickness varies greatly, giving a false impression of sinuosity in LM studies. Length 9–15 μm, width 1–3 μm; density 4800–5000/mm². (*ii*) *O. assmuthi* Holmgren: Rods are generally as in *O. obesus* but much thicker. Length 7–12 μm, width 0.8–5.0 μm, the shape varying considerably; density 4000–7000/mm². (*iii*) *M. gilvus borneensis* Kemner: Hairs numerous, ca. 200–300/mm². Pimpules present all over, rounded; diameter 0.5–4 μm; density ca. 7000/mm². The taxonomic and evolutionary aspects of wing microsculpturing in the Macrotermitinae are discussed.

Key Words: **Wing microsculpturing, Termites, Isoptera, Macrotermitinae,** *Odontotermes, Macrotermes,* **Electron microscope images**

Introduction

The series of earlier light microscope (LM) studies (Roonwal & Co-workers 1967–1983), covering all the major families and over 80 genera and 250 species of termites, showed the occurrence of a dense carpeting of cuticular papillae (in all species) and at least 8 other types of microstructures on wings. (Also see the Introduction in Part I of the present series.)

The current series of scanning electron microscopic (SEM) examination of these microstructures reveal the true and intimate structures. The present part** covers the genera *Odontotermes* and *Macrotermes*.

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** Earlier Parts: I. Roonwal (1985a), Genera *Heterotermes* and *Microtermes*): II. Roonwal, 1985b, Genera *Amitermes* and *Eremotermes*

Earlier (LM) studies on these and other macrotermitine genera are by Roonwal and Chhotani (1967), Roonwal et al. (1980), Roonwal and Rathore (1982) and Roonwal (1983 a, b, summary).

Results

Two species of *Odontotermes* and one of *Macrotermes* were studied.

Genus I. **Odontotermes** Holmgren

(Holmgren, 1912, K. *Svensk. Vet. Akad. Handl.*, Stockholm, **48** (4), p. 35. For other taxonomic details, vide Snyder 1949.)

1. *Odontotermes obesus* (Rambur) (figure 1 and plates 1 & 2)

(*Termes obesus* Rambur, 1842, *Hist. nat. Insects. Neuropteres*, p. 304.)

Material: Imagoes from Jodhpur (Rajasthan, India).

The earlier LM studies showed that, besides several rows of papillae on the margins, both wing surfaces are covered densely with thin, microscopic, subvertically disposed and weakly sinuous cuticular rods (rods of the "*obesus*-type") all over the wing membranes and several rows of tongue-shaped papillae at the anterior (5–6 rows) and posterior (2–4 rows) margins (plate 1). Sizes of papillae 6-8 μm × 5–6 μm; of rods, length 10–15 μm. Density of rods 3966–5550/mm^2. (Roonwal & Chhotani 1967, Roonwal et al. 1980). On the wing scales the papillae are absent, and the rods are shorter (length 3–4 μm) and present in patches (Roonwal & Rathore 1982). Hairs are present in moderate numbers.

The SEM images (figure 1 and plate 2) have now revealed the true and minute structure of the rods which are substraight to slightly bent angularly, rarely shallowly S-shaped. Some are long and thin (length 9–15 μm, maximum width 1–3 μm, the thickness being seldom uniform in a rod and often varying greatly. The edges are sometimes thin and gauzy. The terminal ends are generally rounded, sometimes blunt and truncated, and in a few cases forked. The rods, which had appeared to be sinuous in LM images are really not so, the apparent sinuosity being really due to their varying thickness at different levels. The density of rods, 4800–5000/mm^2, varies with the location on the wing and generally agrees with the earlier LM findings.

Figure 1 *Odontotermes obesus*. Variation in cuticular rods on wing surfaces (outlines from scanning electron micrographs)

2. *Odontotermes assmuthi* Holmgren (figure 2 and plate 3)

(*Odontotermes* (*Odontotermes*) *assmuthi* Holmgren, 1913, *J. Bombay nat. Hist. Soc.* **22** (1), pp 112–113.)

Material: Imagoes from Dehra Dun (U.P., India).

LM studies had shown that on both wing surfaces, besides a few rows of papillae on the margins, there occurs a dense carpeting of minute, subvertically disposed, cuticular rods which are similar to those in *O. obesus* except in being thicker, denser and apparently more sinuous (rods of the "*assmuthi*-

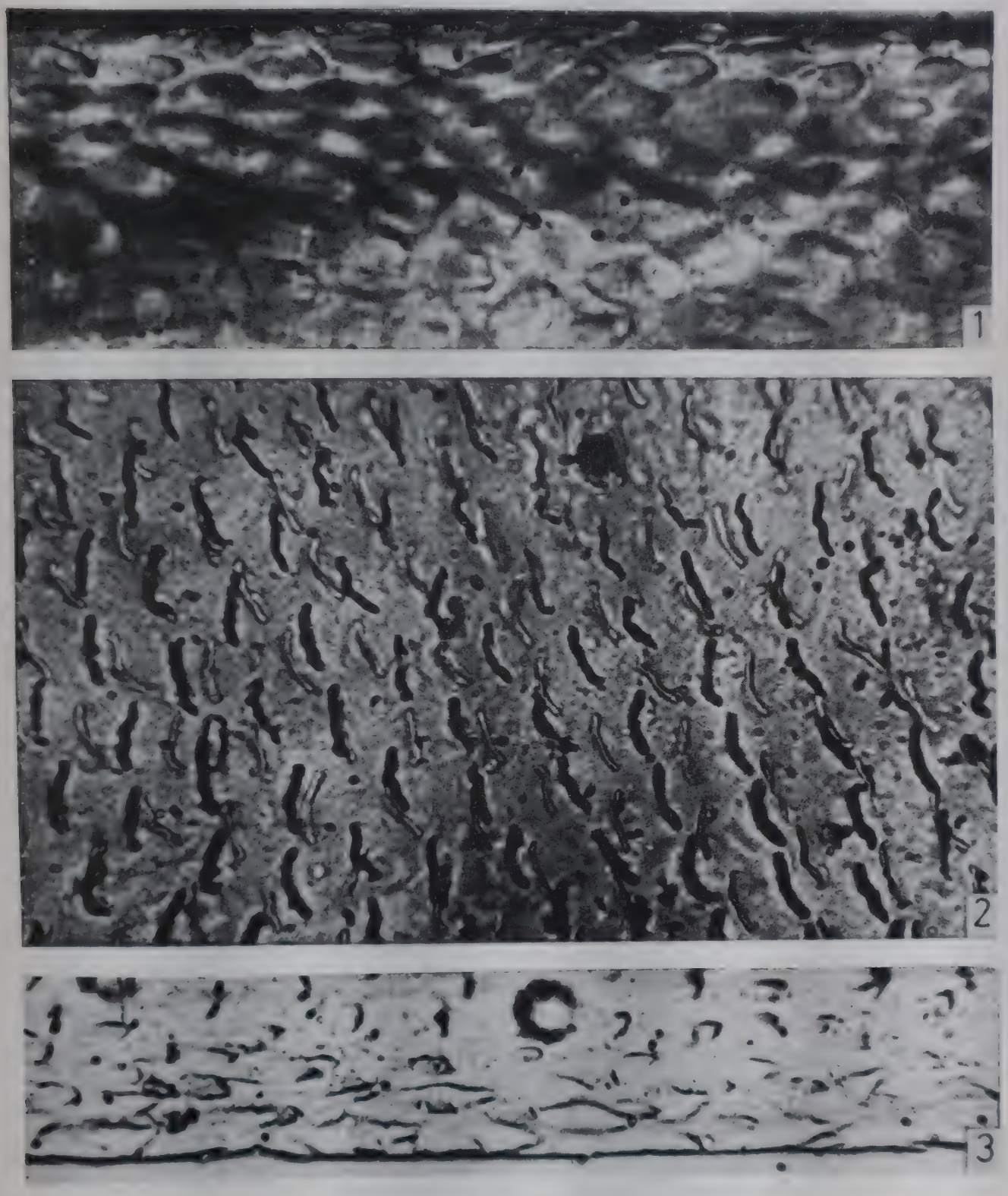

Plate 1 *Odontotermes obesus*. Light microscope photomicrographs of dorsal surface of right forewing, to show microsculpturing. (×700); **1**, Anterior margin, with 5-6 rows of papillae; **2**, Middle of membrane, with cuticular rods; **3**, Posterior margin, with 2-4 rows of papillae

Plate 2 *Odontotermes obesus*. Scan ning electron micrographs of dorsal surface of right forewing in middle of membrane, to show cuticular rods; 1, (× 800); 2, Same, more magnified (× 2400).

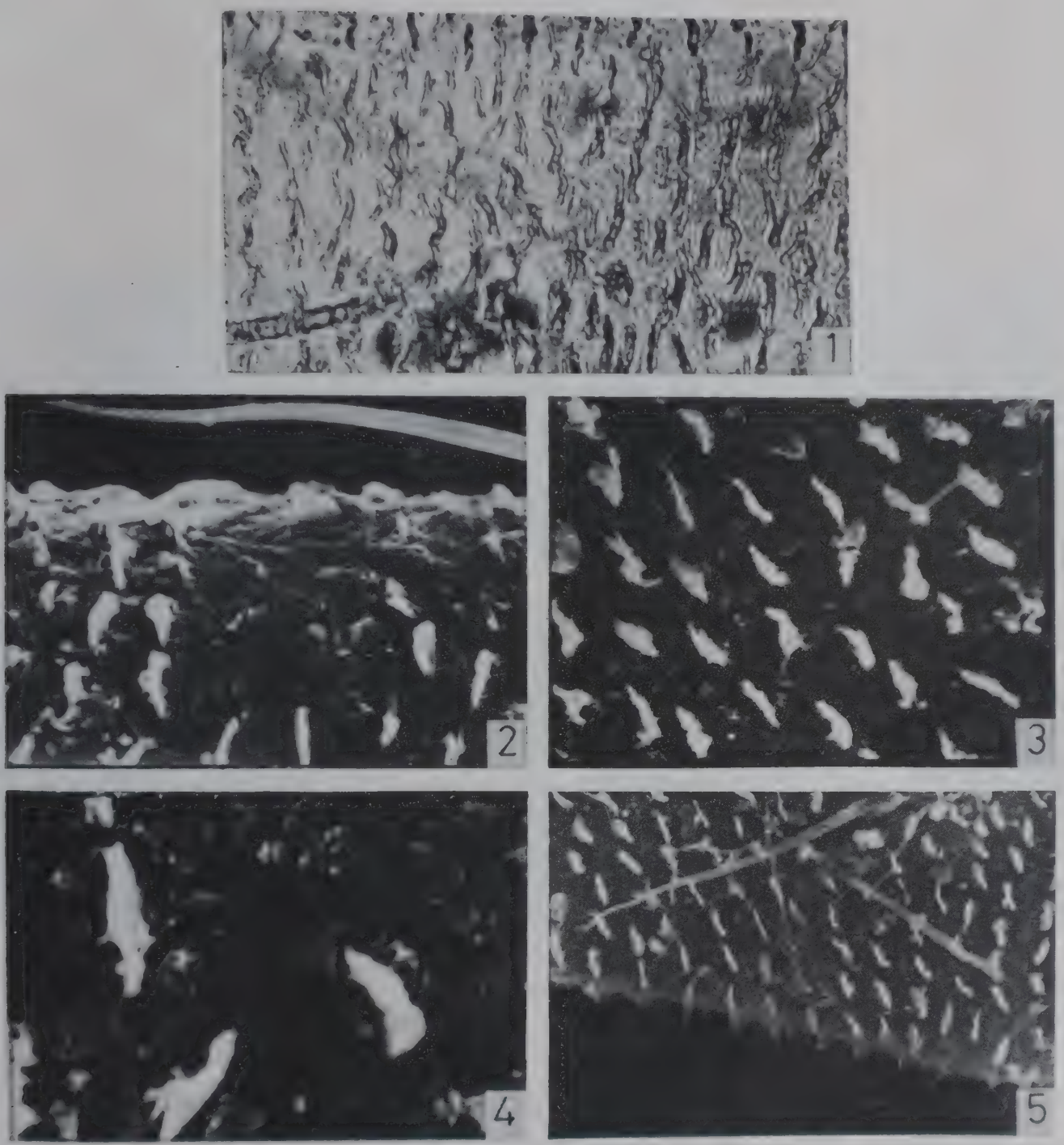

Plate 3 *Odontotermes assmuthi*. Pictures of dorsal surface of forewing, to show microsculpturing. (**1**, light microscope photograph; **2–4**, Scanning electron micrographs.); **1**, Middle of wing membrane. Rods (× 340); **2**, Anterior margin of wing. Papillae on upper edge, and rods below; a long hair is also seen (× 2000); **3**, Middle of membrane. Rods (× 2000); **4**, Same, more magnified. (× 5000); **5**, Posterior margin of wing. Papillae at lower edge and rods elsewhere; two haris are also seen (× 1000)

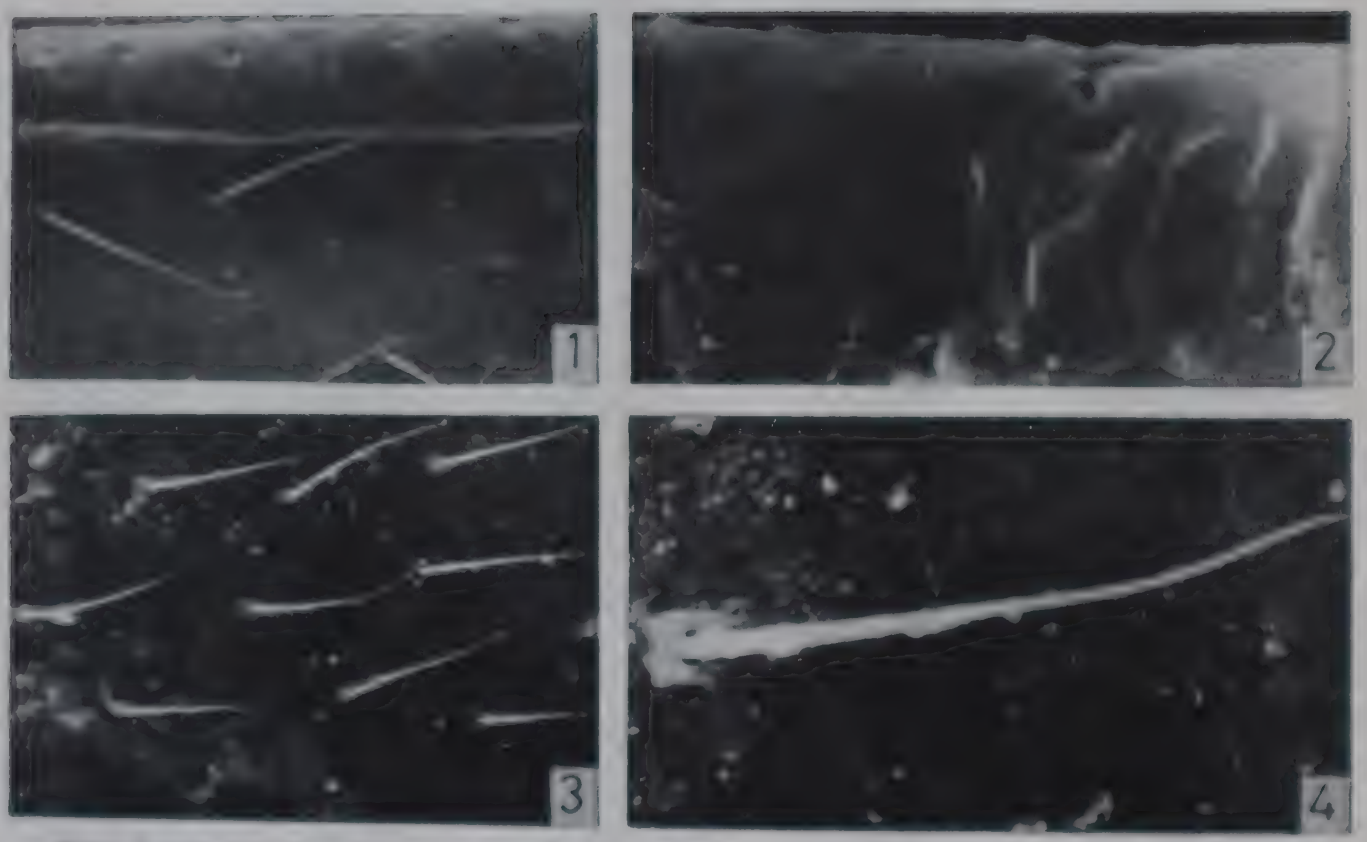

Plate 4 *Macrotermes gilvus borneensis*. Scanning electron micrographs of dorsal surface of forewing, to show microsculpturing: **1**, Anterior margin of wing. Papillae at the edge and hairs below (× 500); **2**, Same, more magnified (× 2000); **3**, On membrane. Hairs and several pimpules (small, rounded, while spots). (× 500); **4**, Same, more magnified. A single hair and a few pimpules seen (× 2000)

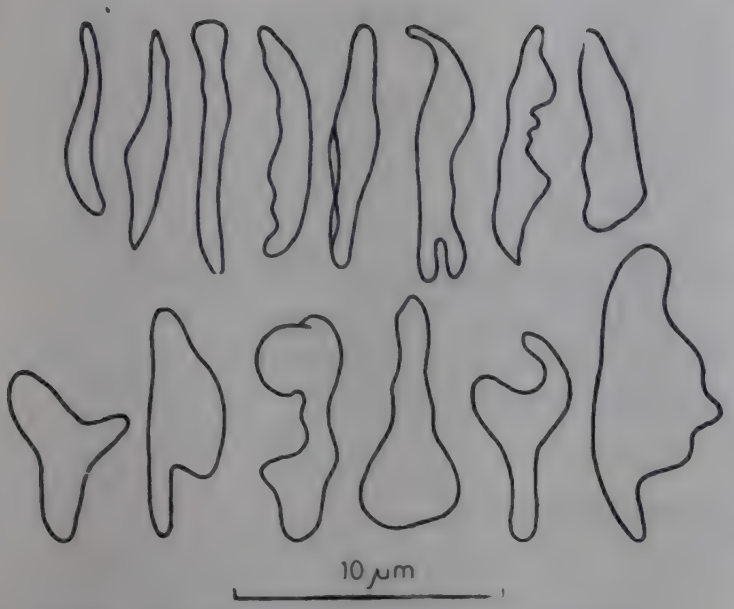

Figure 2 *Odontotermes assmuthi.* Variation in cuticular rods on wing surfaces (outlines from scanning electron micrographs)

type"); hairs are present in moderate numbers (Roonwal & Chhotani 1967, Roonwal et al. 1980, Roonwal & Rathore 1982).

SEM studies have revealed the detailed characteristics of microsculpturing. One or two rows of broad-based, sub-conical papillae are present on the anterior margin and a single row on the posterior. Thick, subvertically disposed rods are present all over the wing surfaces except in the region of the papillae, and are briefly characterised as follows: (*i*) They are shorter, thicker and much stouter (length 7–12 μm, mostly 8–9 μm, maximum width varying widely, 0.8–5.0 μm) than in *O. obesus.* (*ii*) Shapes vary greatly, from substraight rods of nearly uniform, narrow width to those which are considerably widened in an irregular way. (*iii*) As a rule there is no bend and no hint of wavyness, in contrast to the impression given in LM images. (*iv*) The edges are not gauzy (cf. *O. obesus*) and the rods appear to be thicker and more solid. The density of rods varies with the location, from ca. 4000 to 7000 mm^2.

Genus 2. **Macrotermes** Holmgren

(Holmgren, 1911, *K. Svensk. Vet. Akad. Handl.*, Stockholm, **46** (6), p. 11. *Macrotermes* subgen. nov.; no description, but type of subgenus designated as a known species, *Termes carbonarius* Hagen, and this lends it validity. Holmgren 1909, Termitenstudien I, *K. Svensk. Vet. Akad. Handl.*, **44** (3), p. 193, which is given in Synder's world catalogue, 1949, p. 208, as a first reference, does not mention *Macrotermes* at all.)

3. *Macrotermes gilvus borneensis* Kemner (plate 4)

(*Macrotermes gilvus* var. *borneensis* Kemner, 1933, *Mem. Mus. roy. Hist. nat. Belg.*, Brussels, (N S) **4** (8), p. 27)

Material : Imagoes from S E Asia (Sarawak in Borneo Is., E. Malaysia).

LM studies had shown (Roonwal et al. 1980) that both surfaces of wings are densely covered with hairs (length 60–140 μm, density 300/mm^2). Microsculpturing consists of 7–8 rows of papillae on the anterior margin and 2–3 rows on the posterior, as well as numerous minute, rounded pimpules all over.

SEM studies have generally confirmed these findings. They have further shown that the pimpules vary considerably in diameter (0.2–4.0 μm) and their margins are often not smooth and round but rough and frothy. Hairs are present all over in considerable number; length 35–80 μm, density 200–300/mm^2.

Discussion

The SEM studies in *Odontotermes* confirm the presence of marginal papillae and the solid, rod-like nature of the second and more widespread type of microstructures. The apparent sinuous appearance of the rods in LM studies is really due to the varying thickness and irregular shapes of the individual rods. This feature is specially accentuated in *O. assmuthi.*

Four other genera of Macrotermitinae, viz. *Hypotermes, Microtermes, Allodontermes* and *Ancistrotermes*, have, on the basis of LM studies, been found to possess rod-like structures (Roonwal & Chhotani 1967, Roonwal et al. 1980, Roonwal 1981). But

in *Microtermes* the apparently rodlike structures have now, on the basis of SEM studies, been found to be not "rods" but simple, nonasteroid micrasters with a basal, petal-like shroud of gauzy, leafy material and a central, solid, pistil-like rod emerging from the inside of the shroud base (Roonwal 1985a). In *Ancistrotermes* they have already been interpreted as simple micrasters, though their SEM study remains to be made (Roonwal 1981). In *Allodontermes* they were first interpreted as rods (Roonwal et al. 1980), but later on re-interpreted as simple micrasters (Roonwal 1983b, p. 382). *Hypotermes*, which is very close to *Odontotermes* has very similar rods, and it is highly likely that they would prove to be true rods rather than simple micrasters.

Apart from the universal occurrence of papillae, the subfamily Macrotermitinae shows four different types of microsculpturing: (*i*) With pimpules only (*Macrotermes*). (*ii*) With simple micrasters only (*Microtermes*, *Allodontermes*, *Ancistrotermes*). (*iii*) With microsetae only (*Pseudacanthotermes*). (*iv*) With rods only (*Odontotermes*; probably also *Hypotermes*). The absence of uniformity strongly suggests that the Macrotermitinae are a heterogeneous assemlage of genera. It also emphasises the usefulness and reliability of wing microsculpturing as a taxonomic character of considerable value, the more so because it is readily observable.

Acknowledgements

We are indebted to the following persons for assistance with the scanning electron micrographs: To Dr R Guggenheim (Director) and M G Lüönd of the Scanning Microscope Laboratory of the Geological-Palaeontological Institute, University of Basel, Switzerland; and to Dr P K Sen-Sarma (Director of Biological Research) and Mr S S Bisen (S.S.O.) at the Forest Research Institute, Dehra Dun.

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Proc. Indian natn. Sci. Acad. **B51** No. 4 pp 413–456 (1985)

Morphogenesis and Histochemistry of Some Thrips (Thysanoptera: Insecta)-Induced Galls

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A comparative assessment of the developmental morphology of four different types of thrips induced galls *Calycopteris floribundus*, *Loranthus elasticus*, *Pothos scandens* and *Vitis lanceolaria* was made involving the quantification of the degree of hyperplasy, cellular as well as nuclear hypertrophy at different stages of development of the galls compared to those of the normal leaves. Histochemical localisation of proteins, lipids, starch, condensed tannin precursors, tannin, polyphenol oxydase, peroxidase and acid phosphatase in the gall tissues in comparison with those in the normal leaves was also analysed. Results indicated a greater localisation of proteins, polyphenol oxidase, peroxidase and acid phosphatase in gall tissues, especially in the nutritive zone than in the normal leaf tissues. A hyperactivity of tannin in the gall tissues as compared to the normal leaf tissues was notable. A functional elaboration in the cells closer to the feeding sites of the cecidozoan and an elaboration in terms of the morphological criteria of the cells farther away from the insect feeding site has been established.

Key Words: Morphogenesis, Histochemistry, Developmental anatomy, Thrips, Galls

Introduction

Investigations on the developmental morphology and comparative anatomy of thrips galls were initiated by Karny and Docters van Leeuwen (1913) on different types of thrips-induced galls from Java, followed by the contributions of Ananthakrishnan (1978) and Raman and Ananthakrishnan (1983) on a number of thrips galls from India. Further contributions towards studies on morphological variations in the ceratonean galls of *Schefflera racemosa* (Krishnamurthy et al. 1975), morphology and histology of the leaf galls of *Cordia obliqua*, induced by *Aneurothrips priesneri* Bhatti. (Krishnamurthy et al. 1977) and of *Casearia tomentosa* Roxb. induced by *Gynaikothrips flaviantennatus* (Raman et al. 1978) as well as on the leaf fold galls of *Maytenus senegalensis* (Lam.) Excell., induced by *Alocothrips hadrocerus* (Karny) (Raman & Ananthakrishnan 1979) have further highlighted the principal cecidogenetic phenomena involved in gall development. Assessing the developmental and comparative morphology of some thrips-induced galls,

Raman and Ananthakrishnan (1983a) have provided the ultrastructural details of thrips galls confirming the presence of nutritive zone in those galls. Recently, Ananthakrishnan (1983) has reviewed some of the specialised strategies displayed by gall thrips in cecidogenous systems.

Against this background an attempt has been made to make a comparative assessment of the developmental morphology and histochemistry of the galls on *Calycopteris floribundus* Lamk., *Loranthus elasticus* Desb., *Pothos scandens* Linn., and *Vitis lanceolaria* Roxb., in relation to the degree of cellular hyperplasy, hypertrophy and nuclear hypertrophy which result in considerable variations in form and organization.

Material and Methods

Galls in various stages of development were collected from their natural habitat. Depending on their relative dimensions and approximate age in terms of days, supplemented further by population counts of the inhabiting thrips species, the galls were graded into various developmental stages as young, mature and old; normal leaves and axillary buds (in *Calycopteris*) of comparable age were also obtained for the investigation.

Normal and gall tissues were fixed in Formal-Acetic-Alcohol (FAA) and also 5% Gluteraldehyde in phosphate buffer (7.2 *p*H). After an initial fixation for 24–48 hr in buffered gluteraldehyde, the material was washed repeatedly in the phosphate buffer (7.2 *p*H) and in double-distilled water. They were then passed through the Ethanol-TBA series for dehydration and clearing (Sass 1958) and embedded in paraffin wax. The blocks were cut at 7–10 μm range for FAA-fixed material and at 4–7 μm for gluteraldehyde-fixed material. Serial sections were sequentially numbered and stained with safranin-toluidine blue (1%) combinations. Photomicrographs were taken with a WILD MPS 15/11 Semiphotomat with interchangeable 35mm magazine (camera factor of 0.32×), using Leitz Dialux 20 Microscope and Wild Zoom Stereomicroscope.

For studying the vasculature patterns of *Calycopteris* galls, entire galls in varying ages were cleared initially in 30% ethanol and further in lactophenol and 10% KOH, and tested with lignin-sensitive phloroglucinol.

Measurements of the degree of cellular hypertrophy were made using a gauged micrometer. Nuclear volume in both the normal and galled tissues were calculated using the formula "4/3 abc π", following the method described by Hesse (1968). Relative dimensions of the cells and nucleus in normal and abnormal tissues were statistically analysed (up to 1% level) using the T-test of significance.

Histochemical localisations of lipids, proteins, starch, tannin, condensed tannin precursors, and also enzymes such as acid phosphatase, polyphenol oxidase, and peroxidase were carried out in normal and gall tissues. Histological preparations of the galls and the normal plant organs (Axillary buds and associated primordial leaves in *Calycopteris* and leaves in *Pothos*, *Loranthus* and *Vitis*) of comparable age (table 1) were histochemically analysed. The qualitative increase or decrease in the enzymes and other compounds studied were assessed in terms of intensity of staining and the degree of distribution in tissues.

Proteins: Proteins were localised using filtered Amido Black stain (Weime 1959). Fresh, hand sections of the normal and galled leaves were stained for 2–3 min and were washed in 2% (V/V) acetic acid for 5 min and later in distilled water. The stained material was mounted temporarily in glycerine jelly and immediately photographed. Proteins gave a blue-colour reaction.

Lipids: Sections were placed in ethylene

glycol for 3-5 min and subjected to occasional shaking after which these were transferred to a Sudan III dye (Chiffelle & Putt 1951). After thorough washing for 3-5 min in distilled water, the sections were mounted in glycerine. Fats, oils, and waxes stained orange.

Starch: (IKI-reaction, Johanson 1940) Sections were kept in IKI solution (Iodine, 02 g; KI, 2g; distilled water, 100ml). The starch appeared blue to black within a few minutes.

Taninns: Sections were treated in Lugol's Iodine solution (Iodine, 4g; KI, 6g; distilled water, 100ml); to this was added a drop of 10% ammonium hydroxide. Tannins gave a bright red colour.

Condensed tannins precursors: Sections were placed in DMB reagent (1% 2, 4-Dimethoxy benzaldehyde in 95% ethanol and 18% HCl) (Mace & Howell 1974) and incubated for 5 min at 23°C. These were then placed in a drop of DMB Reagent in a glass slide and photographed immediately. Condensed tannin precursors gave purplish red colour reactions.

Acid phosphatase: This was localised by a slight modification of the procedure suggested by Gomori (1952). Sections were treated in a substrate solution ($PbNO_3$, 0.6 g, 0.5M acetate buffer (*p*H 5.5), 500 ml) 0.1 M sodium glycerophosphate 50 ml. The *p*H was adjusted to 5.0. The sections were incubated in the substrate solution for 1–2 hr at 37°C, subsequently rinsed in distilled water and then placed in a dilute ammonium sulphide solution (1–2ml in a coplin jar of water) for 5 min. They were then rinsed and mounted in distilled water and immediately photographed. Control tests were done (*i*) by incubating the tissues in a medium minus substrate, and (ii) by directly placing the tissues in the ammonium sulphide solution without being placed in a substrate solution. Acid phosphatase appeared as black deposits.

Peroxidase: Sections were placed in an incubating solution composed of 0.1M phosphate buffer (*p*H 4.5), 0.1M H_2O_2 and approximately 0.01M Benzidine (Isaac & Winch 1947). The concentration of benzidine was not critical. Controls were incubated without H_2O_2. The localisation of peroxidase activity was indicated by a blue to brown colour.

Polyphenol oxidase: Sections were incubated in the freshly prepared medium 50 mg of D, 1,3,4-Dihydroxy phenyl alanine (DOPA) dissolved in 10ml of 67mM Sorensen's phosphate buffer (*p*H 7) (Sexton & Hall 1978), for 1 hr at 37°C. Controls were incubated without DOPA. The brown colour indicated localisation of polyphenol oxidase.

Fresh, and 70% alcohol-preserved, normal and gall material (2 × 2mm²) were dried in dust-free desiccator for 2–3 days. These were fixed to aluminium stubs using double adhesive tape strips and then coated with gold for 2–3 min in a standard ion coater. Micrographs were made using Hitachi Scanning Electron Microscope of Table Top Model S 415A under 15 KV emission current.

Results

Developmental Morphology

(*a*) *Calycopteris floribundus*

The pouch galls of *Calycopteris floribundus* (plate 1) induced by *Austrothrips cochinchinensis* are generally found in the axillary positions and are distributed in almost all

Table 1 *Age of the normal and galled leaves*

Host plant	Young	Mature	Old
Calycopteris floribundus	4–8	25–30	70–80
Loranthus elasticus	3–5	16–20	25–30
Pothos scandens	2–4	12–16	20–23
Vitis lanceolaria	2–5	10–15	20–25

parts of Kerala, Karnataka, and in some parts of Tamil Nadu. When fresh shoots emerge after the monsoon, galls develop on axillary buds, and rarely on terminal buds. Well-developed galls are globose, unilocular, irregular with highly wrinkled and convoluted surface reaching 5–8 cm in length and 3–4 cm in width (plates 1A–F), the hollow, globose-gall having an ostiole at the terminal region between the rudimentary leaves. The outer and the inner sides of the gall have densely distributed hairs, the densest area being the ostiolar region. In developing galls, generally 2-3 adult thrips occur, laying their eggs on the trichomatous wall. With the development and maturity of galls, the population of thrips increases to 2000–3000 individuals.

Gravid female thrips infest the axillary buds when they attain a size of 0.5–1.0 cm. The axillary buds possess leaf primordia arranged in opposite decussate manner around the developing shoot apex. Thrips feed on the superficial layers of the apical meristem and as a result of the feeding damage by *A. cochinchinensis* the apical meristem is destroyed (plates 2A–H). Simultaneously, thrips feed along the marginal portions of the leaf primordia from the basal region upwards. Feeding injury on 1-2 epidermal cells and primordial mesophyll cells triggers the proliferation of the tissues and evokes wound healing responses, resulting in the fusion of the two oppositely placed young leaf primordia as well as the two leaf primordia of the succeeding node (figures 1A–H).

The surface of normal foliage and galls show unicellular hairs particularly along the adaxial surfaces (plate 3D) and stellate hairs (plates 3A & C) on the abaxial surfaces with a number of stomata distributed among the hairs. The inner surface of the galls has elongated unicellular trichomes (plate 3E) as also along the adaxial surface of the normal leaves. The galls generally show a denser distribution of hairs than on the normal leaves. The outer surfaces of galls display a number of unicellular hairs along with the stellate hairs (plate 3B) —the ventral surface shows only stellate hairs. Unicellular hairs together with stellate hairs also occur on the abaxial surface of young leaves, though not as dense as in galled leaves. These patterns are suggestive of the developmental sequence of the gall, namely that the adaxial surface with long unicellular hairs forming the inner surface of the gall and abaxial surface forming the outer surface characterised by unicellular and stellate hairs.

The highly convoluted and wrinkled walls of *Calycopteris* galls show 21–34 parenchyma cell layers in the young galls, 40–52 in mature galls, and 60–84 in old galls (plates 4B, F & G). The differentiation of the parenchyma cells into tracheary elements is common in the gall tissue (plate 4C) together with the excessive vascular proliferation. Young galls show parenchyma cells with dense cytoplasm constituting the nutritive zone (Plate 4E). Deposition of calcium oxalate crystals in cells away from the nutritive zone is an unusual phenomenon not seen in the normal leaves. Continued feeding of thrips populations sometimes results in extensive wall growth of 70–90 cell layers towards the interior of the gall chamber providing only a limited space within the galls (plate 2 G) restricting the mobility of the inhabiting thrips. As the gall matures, gall cells along the periphery exhibit hypertrophy resulting in lateral stretching of the cell (plate 4 G). Significant nuclear hypertrophy is evident in the gall tissues, unlike in the normal leaves, the maximum enlargement of nuclei occurring in old galls. Nuclear volume in gall tissues increases by 3.63 times that of the normal tissues (table 2). Cellular hyperplasy is also at its maximum in these galls, whereas cellular hypertrophy constituting maximum lateral stretching is highly

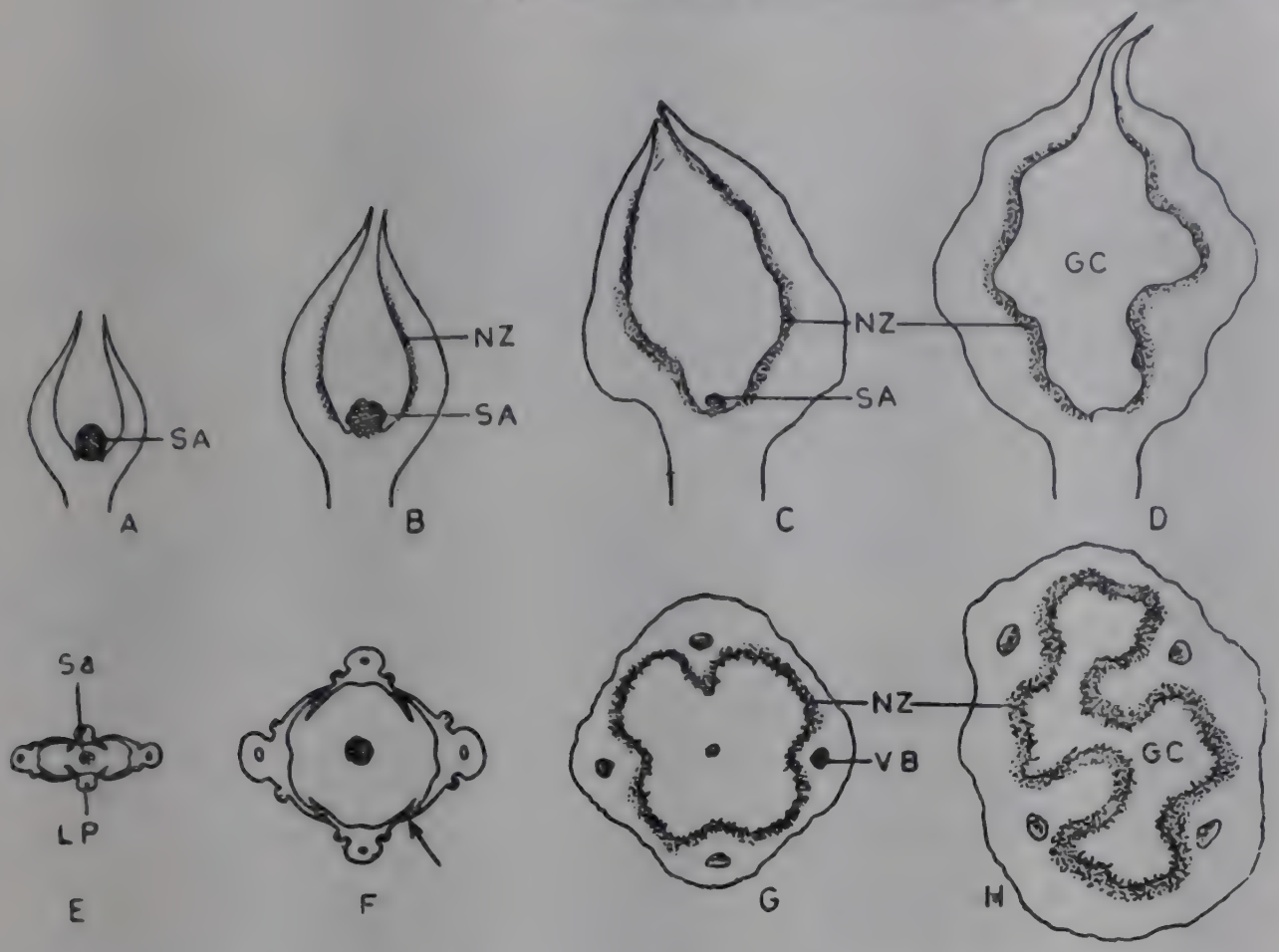

Figure 1 *Calycopteris floribundus;* **(A)** Axillary bud (LS); **(B–D)** Gall (LS)—Developmental stages showing the gradual disintegration of the shoot apex as a result of thrips feeding; **(E)** Axillary bud (TS); **(F–G)** Gall (TS) Developmental stages showing fusion of the leaf primordia
CG, Gall chamber; **LP,** Leaf primordia; **NZ,** Nutritive zone; **SA,** Shoot apex; **Sa,** Shoot axis; **VB,** Vascular bundle

significant in young galls and horizontal stretching of the cells is maximum in mature and old galls (table 2). These types of horizontal and lateral stretching of the cells are maximum in cells away from the nutritive zone with a gradual decrease towards the nutritive cells. The nutritive tissues showed dense cytoplasm, prominent nuclei, nuclear hypertrophy and split vacuoles. Table 2 shows the comparative rates of cellular hyperplasy, hypertrophy, and nuclear hypertrophy at different ages of the galls compared with the normal ones.

(*b*) *Loranthus elasticus*

Continued feeding of *Phorinothrips loranthi* on undifferentiated leaves of *Loranthus elasticus* results in the rolling of leaf margins on either side towards the midrib causing epiphyllous rolls (plate 5D). The galled leaves appear highly wrinkled and corrugated. Occasionally 'partial' galls with laminar rolling towards the midrib region seen only on one side are also formed.

The dorsiventrally differentiated mature normal leaves of *L. elasticus* show two well-organised layers of palisade cells and 3–4 layers of closely arranged spongy parenchyma cells. Sclerenchymatous idioblasts occur distributed throughout the lower mesophyll cells.

Owing to the feeding injury caused by *P. loranthi* along the adaxial side of differentiating laminae, 2-3 epidermal cells and primordial palisade cells are damaged, triggering off a series of wound healing responses characterised primarily by meristematic activity in

Table 2 *Cellular hypertrophy, hyperplasy and nuclear hypertrophy in* Calycopteris floribundus

Age of the normal and galled leaves		Cellular hypertrophy				Cellular hyperplasy		Nuclear hypertrophy	
		Lateral stretching		Horizontal stretching		Number of cell layers		No. of times increase in nuclear volume in gall when compared to normal ones	
		Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value
	Young normal	9.98±0.19 (6.96—11.6)		7.65±0.15 (4.64—9.28)		6.5±0.01 (6—7)			
8—12 days	leaf		13.44*		10.26*		7.34*		11.03*
	Young gall	28.30±0.44 (23.2—34.8)		36.86±0.52 (32.48—46.4)		27.0±0.25 (21—34)		3.15±0.08 (1.83—4.44)	
	Mature normal	29.41±0.52 (20.2—36.8)		13.22±0.19 (11.6—16.24)		10.5±0.01 (10—11)			
25—40 days	leaf		4.59*		14.70*		8.56*		11.09*
	Mature gall	38.98±0.49 (32.48—41.76)		39.44±0.39 (32.48—46.4)		46.0±0.33 (40—52)		3.86±0.11 (2.67—6.67)	
	Old normal	33.82±0.61 (20.38—41.76)		17.40±0.50 (11.6—25.52)		11.0±0.02 (10—12)			
70—90 days	leaf		1.49^{NS}		14.51*		11.86*		11.87*
	Old gall	38.28±0.50 (34.8—46.4)		64.07±0.80 (39.44—74.24)		72.0±0.43 (60—84)		4.14±0.12 (2.60—6.00)	

*Significant at 1% level = 2.552

^{NS}Non significant

Values in the parenthesis indicate range.

Plate 1 (A—F) Pouch galls of *Calycopteris floribundus* induced by *Austrothrips cochinchinensis*–Developmental stages

Plate 2 *Cal[illegible] fumbundus*. (A) Young gall (LS)—Initial damage on the shoot apex due to thrips feeding. (B) Young gall (LS)—Shoot apex with damaged cells, arrow—dividing meristematic tissues. (C) Young gall (LS)—Damaged shoot apex. (D) Shoot apex (LS)—Dividing meristematic tissues. (E) Mature gall (LS)—Damaged shoot apex with necrosed cells. (F) Young gall (LS)—Degenerating cells of shoot apex. (G) Mature gall (LS)—Arrow—Damaged shoot apex, hyperplasied cells in the walls; (H) Old gall (LS)
FD—Feeding damage; NZ—Nutritive zone; SA, Shoot apex; S—Shoot apex damage

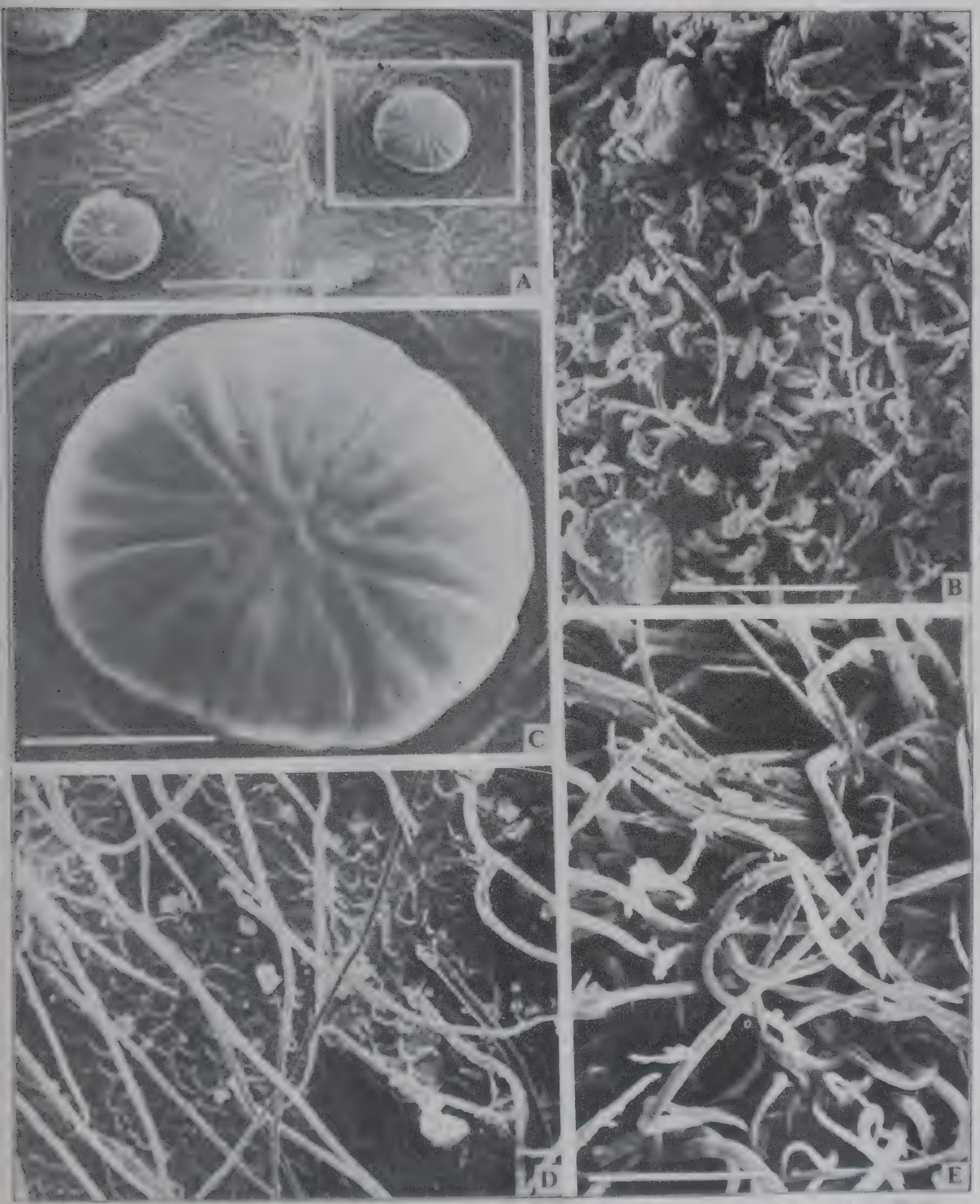

Plate 3 *Calycopteris floribundus*. (**A** and **C**) Mature leaf abaxial surface with steliate hairs (Scale: A—15K 20× 4NM; C—15K 20×4NMB; (**B**) Mature gall outer surface with unicellular and stellate hairs (Scale: 15K× 4NM); (**D**) Mature leaf adaxial surface with unicellular trichomes (Scale: 15 15×4NM); (**E**) Mature gall inner surface with unicellular trichomes (Scale: 15 12×4NM)

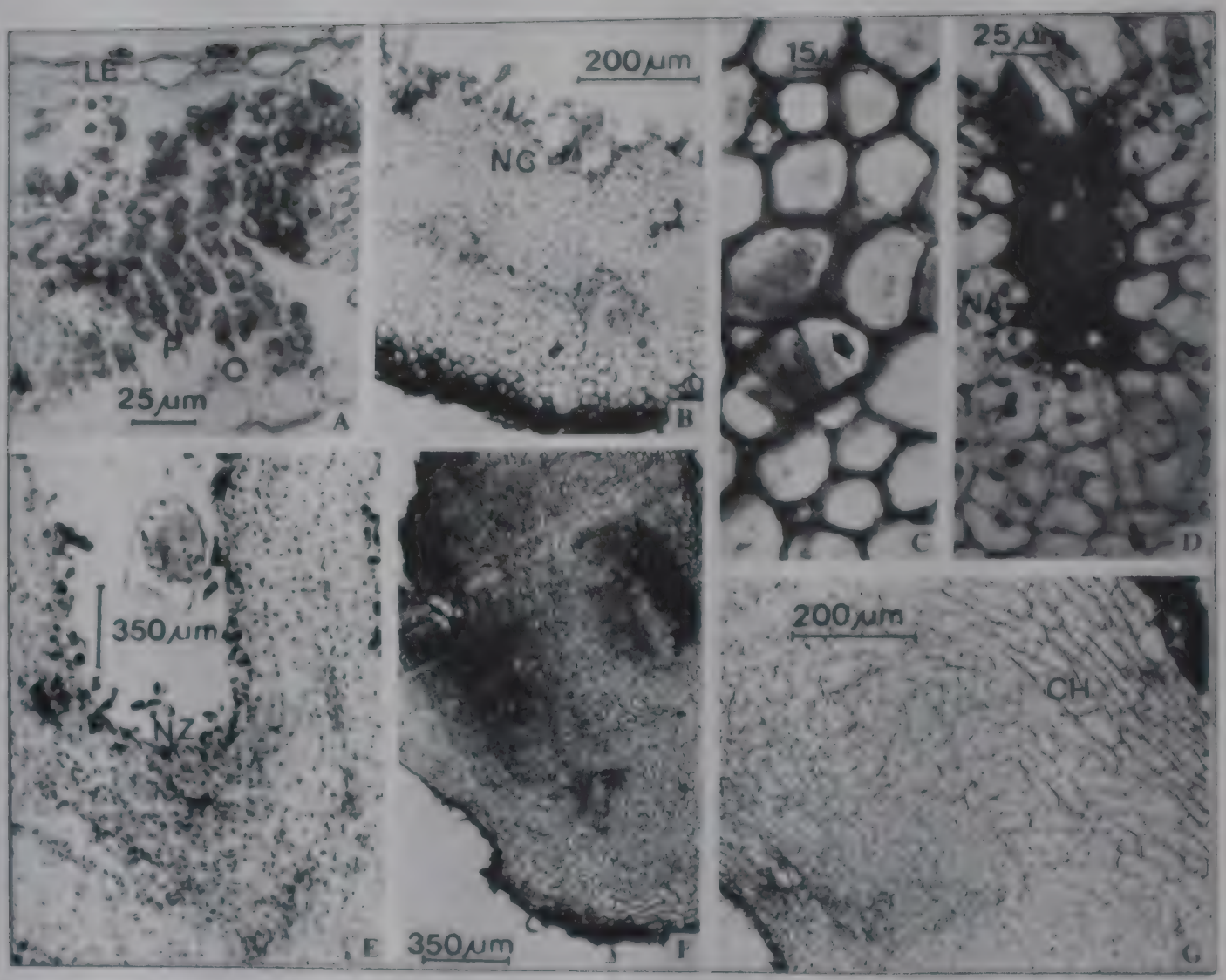

Plate 4 *Calycopteris floribundus*. **(A)** Normal mature leaves (TS)—Palisade and spongy mesophyll cells; **(B)** Mature gall (TS)—Hyperplasied cells; **(C)** Parenchyma cells—Redifferentiation to xylary elements; **(D)** Mature gall (TS)—Hyperplasied and necrosed cells; **(E)** Mature gall (TS)—Nutritive zone; **(F and G)** Old gall (TS)—Hyperplasied nutritive zone cells and hypertrophied cells farther away

CH, Cellular hypertrophy; **LE**, Lower epidermis; **NA**, Necrosed area; **NC**, Nutritive cell; **NZ**, Nutritive zone; **P**, Palisade parenchyma; **S**, Spongy parenchyma

Plate 5 (A—C) *Pothos scandens*—Hypophyllous roll galls on the leaves induced by *Tetradothrips foliiperda;* (D) *Loranthus elasticus*—Epiphyllous roll galls on the leaves induced by *Phorinothrips loranthi;* (E) *Vitis lanceolaria*—Hypophyllous leaf roll galls induced by *Liothrips viticola*

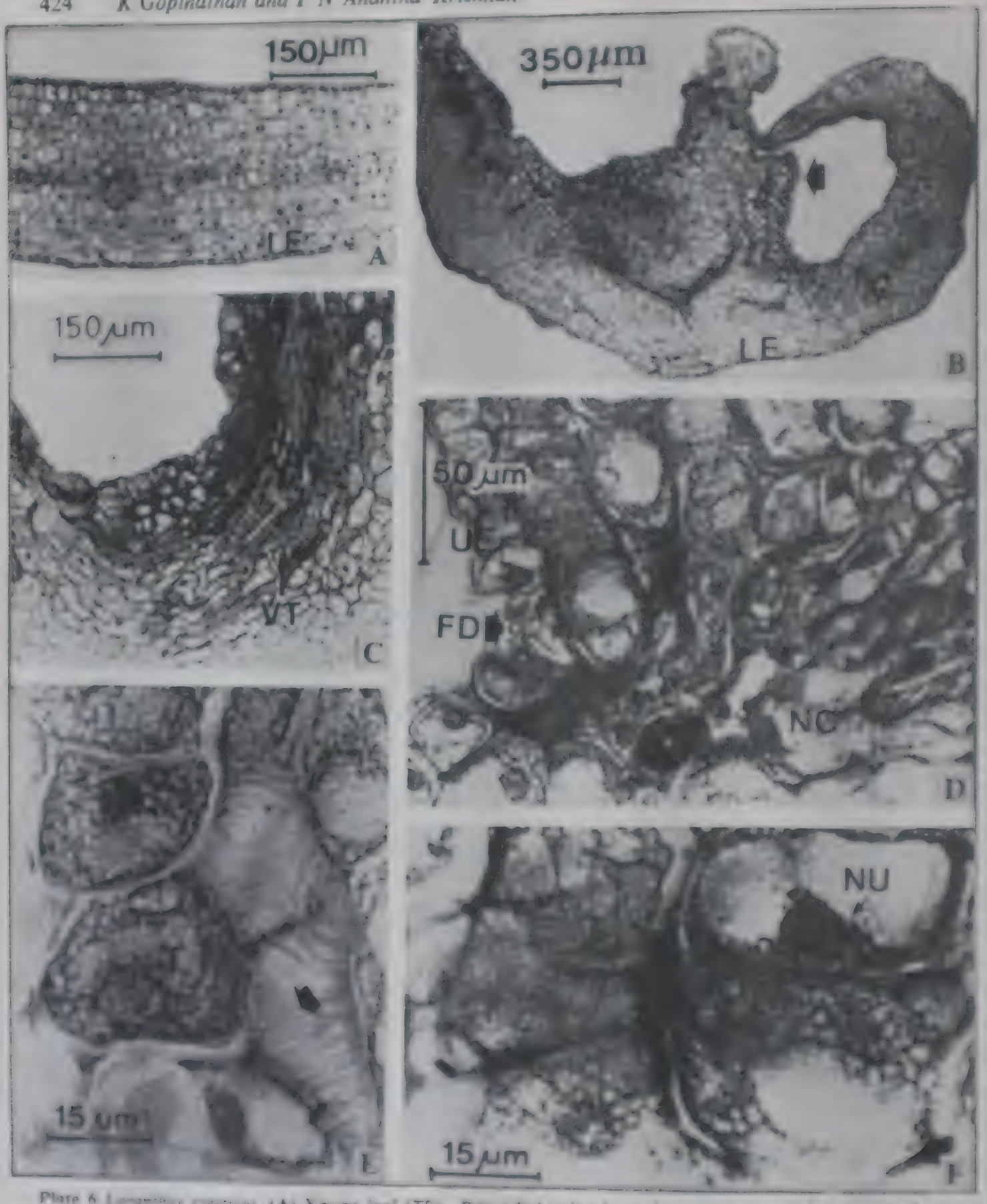

Plate 6 Longitudinal sections. (A) Young leaf (TS)—Primodial palisade and spongy mesophyll cells. (B) Young gall (TS)—Hyperplasied cells in the midrib and laminar portions; (C) Mature gall (TS)—Vascular traces in the nutritive zone. (D) Young gall (TS)—Hyperplasied and hypertrophied nutritive zone cells. (E) Young gall (TS)—Arrow—transdifferentiation of parenchyma cells to xylary elements. (F) Young gall (TS)—Nutritive cells with hypertrophied nuclei and vacuoles.

FD, Feeding damage; LE, Lower epidermis; NC, Nutritive cells; NU—Nucleus; UE, Upper epidermis; VT, Vascular traces.

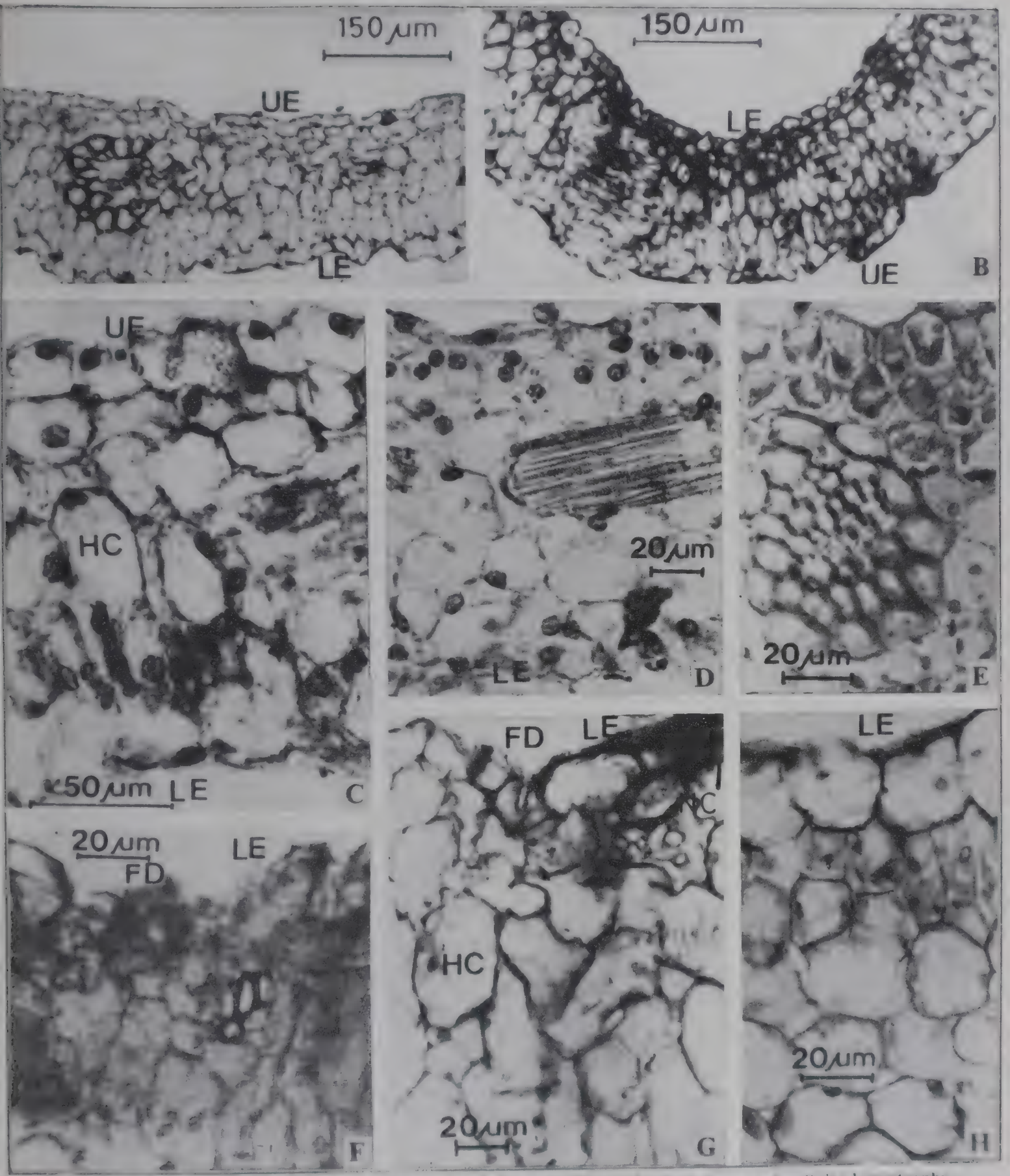

Plate 7 *Pothos scandens*. (A) Mature leaf (TS); (B) Young gall (TS)—Nutritive zone and cellular hypertrophy in the spongy mesophyll cells; (C) Mature gall (TS)—Densely cytoplasmic cells and hypertrophied nuclei; (D and E) Mature leaf (TS)—Palisade cells and spongy parenchyma cells with raphides; (F) Young gall (TS)—Feeding ruptures near the vascular bundles; (G) Mature gall (TS)—Feeding damage, necrosed and hypertrophied cells; (H) Old gall (TS)—Hypertrophied epidermal and palisade cell layers

FD, Feeding damage; **HC**, Hypertrophied cell; **LE**, Lower epidermis; **NC**, Necrosed cell; **UE**, Upper epidermis

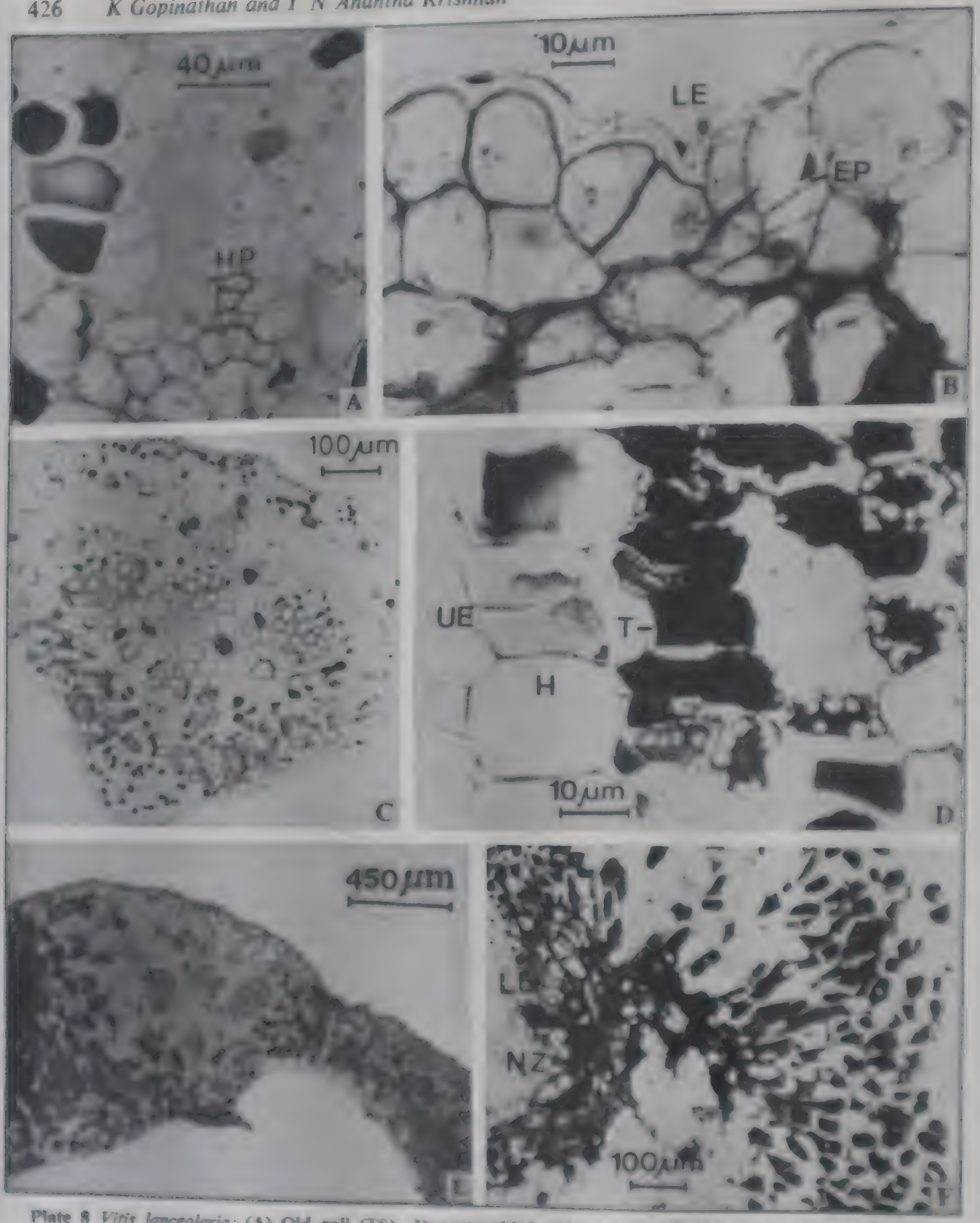

Plate 8 *Vitis lanceolaria*: (A) Old gall (TS)—Hypertrophied phloem cells. (B) Mature gall (TS)—Epidermal proliferation and irregular palisadisation. (C) Mature leaf (TS)—Midrib region. (D) Old gall (TS)—Hypertrophied hypodermis and vacuolated tanniniferous cells away from the nutritive zone. (E) Old gall (TS)—Hyperplasied parenchyma cells in the midrib region with laterally pushed vascular bundles. (F) Old gall (TS)—Nutritive zone with necrosed and tanniniferous cells.

EP, Epidermal proliferation. **H**, Hypodermis; **HP**, Hypertrophied phloem. **LE**, Lower epidermis; **NZ**, Nutritive zone; **T**, Tannin; **UE**, Upper epidermis.

the affected zone (plate 6 D). The proliferation patterns are distinct in that periclinal divisions occur in primordial palisade cells and anticlinal divisions in the spongy mesophyll cells. While this proliferation pattern contributes to the expansion of the lamina in the transverse axis, further divisions in both periclinal and anticlinal axes and occasionally irregular diagonal divisions (plate 6D) in the lower mesophyll cells facilitate the increase in thickness of the lamina (plate 6B). The cecidogenetic influence has a varied impact on the leaf tissues in the process of galling so that the spongy mesophyll cells show hypertrophy, especially in the transverse axis of cells, facilitating the upward rolling of the lamina.

Young galls show 16–20 layers of cells as against the 7–9 layers of cells in normal leaves of corresponding age. Proliferation is evident in the upper epidermal cells as isolated patches near the feeding sites. With the maturation of the gall, there is an increase from 6–8 (in young galls) to 10–12 (in mature galls) in the nutritive cell layers. The cells of this specialised, functional zone are characterised by dense cytoplasm, prominent hypertrophied nuclei and numerous large and small vacuoles (plate 6F). The nuclear volume shows a gradual increase from the innermost cell layer (nutritive zone cells) to the 6th outerlying layers of cells and a gradual decrease from the 7th layer onwards. It is significant that this feature appears identical in young, mature, and old galls (figure 2). As a result of galling, the affected leaf shows proliferation of vascular traces through dedifferentiation of parenchyma cells, the maximum degree of ramification being in old galls (plates 6C & E). In mature and old gall tissues the spongy mesophyll layers constitute compactly arranged plasmolysed parenchyma cells. These parenchyma cells away from the nutritive zone are sparsely cytoplasmic (plate 6B). As the gall matures, the

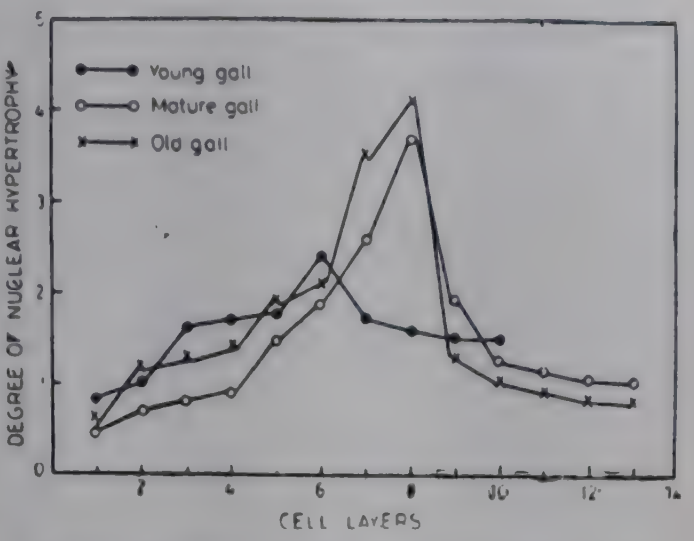

Figure 2 Degrees of nuclear hypertrophy in young, mature and old gall tissues of *Loranthus elasticus*

tissues show more tanniniferous cells, the maximum being found in old galls.

The sclereid-idioblast differentiation, evident in mature and old normal leaves, is almost absent in gall tissues except one or two isolated elements along the lower mesophyll region rarely. In old gall tissues in specific areas, the lower mesophyll cells show disintegration profiles and the cells surrounding these areas are fast-staining, showing several transversely divided cells.

Varying thrips populations cause the formation of 'partial' galls involving the rolling of only one of the halves of the laminar blade as well as 'complete' galls where both the laminae roll towards the mibrib region. Such a variation in gall formation depends mainly on the stage of developmental sequence of the leaf primordia and the size of the thrips populations. A highly significant increase of 1.51 times the nuclear volume of normal leaves is evident in gall tissues (table 3). Horizontal stretching of the cells is highly significant in mature galls and old galls whereas lateral stretching is seen only in young galls (table 3). Cellular hyperplasy is also observed even from the younger stages of development. Table 3 shows the degree of hyperplasy and hypertrophy in young, mature, and old galls compared to the normal

Table 3 *Cellular hypertrophy, hyperplasy and nuclear hypertrophy in* Loranthus elasticus

Age of the normal and galled leaves (days)		Cellular hypertrophy				Cellular hyperplasy		Nuclear hypertrophy	
		Lateral stretching		Horizontal stretching		Number of cell layers		No. of times increase in nuclear volume in gall when compared to normal ones	
		Mean ± SE	*t* test value	Mean ± SE	*t* test value	Mean ± SE	*t* test value	Mean ± SE	*t* test value
3–5	Young normal leaf	23.43±0.29 (20.38—27.84)	2.89*	21.19±0.31 (16.24—25.52)	3.46*	8.0±0.12 (7—9)	3.24*		3.15*
	Young gall	29.23±0.52 (23.2—39.44)		48.49±1.05 (25.52—56.00)		18.0±0.20 (16—20)		1.51±0.05 (0.85—2.35)	
16–20	Mature normal leaf	26.91±0.25 (23.2—30.16)	1.57^{NS}	21.80±0.22 (18.56—23.2)	3.73*	10.0±0.02 (9—12)	3.53*		16.32*
	Mature gall	24.07±0.48 (18.56—34.8)		48.65±1.29 (22.52—57.04)		22.0±0.21 (18—28)		1.57±0.10 (0.51—3.69)	
25–30	Old normal leaf	29.23±0.35 (25.52—34.8)	1.20^{NS}	27.84±0.41 (23.2—32.48)	3.88*	13.0±0.01 (12—14)	3.64*		4.58*
	Old gall	32.71±0.79 (23.2—41.76)		50.12±1.40 (34.8—64.96)		24.0±0.31 (18—30)		1.47±0.05 (0.58—2.37)	

*Significant at 1% level=2.552; ^{NS}, Non significant
Values in the parenthesis indicate range

tissues and also the degree of hypertrophy in the nucleus. Excessive cellular hypertrophy in the periclinal (=horizontal) axis, the maximum being in cells away from the nutritive tissues is noticed in all the three stages of the galls.

(*c*) *Pothos scandens*

Tetradothrips foliperda caused hypophyllous roll galls on *Pothos scandens*; the leaf margins roll from one edge to the other (plate 5A–C) with feeding spots all over the laminar portions, reddish to purple in young galls and yellowish to white in mature galls (plate 5B & C). The winged lower portions of the leaves are also affected.

Young leaves show undifferentiated primordial mesophyll cells. Dorsiventrally differentiated mature leaves have two layers of densely packed chlorophyllous palisade parenchyma cells and three layers of spongy parenchyma cells (plates 7D & E). Crystals are deposited in the form of raphides and druces in epidermal and spongy mesophyll cells (plate 7D).

The feeding damage is often restricted to the epidermal cells. Cross sections of the young galls show the presence of feeding ruptures nearer the vascular bundles (plate 7F), which are never found on the cells with calcium oxalate crystals or anywhere near them. Feeding results in the rupture of certain cells of the lower epidermis as well as 2-3 cells of the primordial spongy mesophyll cells (plates 7F & G). As a result of feeding, vertical divisions take place in the primordial palisade cells of the young galls away from the feeding spot while only stretching in the vertical axis occurs in the primordial spongy cell layers (plate 7B). Cells adjacent to the feeding regions are non-vacuolated and densely cytoplasmic, and bear hypertrophied nuclei (plate 7C).

Hypertrophy is more prominent in mature galls, being confined to the anticlinally dividing cells of the palisade mesophyll cell layers and upper epidermal cells. There is no increase in the number of cell layers. A well defined nutritive zone cannot be detected in the gall tissues since all the cell layers take a lighter staining than the cells of the nutritive zone in the galls of *Loranthus*, *Vitis* and *Calycopteris*. In old galls, hypertrophy is evident in all the cell layers the maximum being in the upper epidermal and palisade cell layers, away from the feeding zone (plate 7H). The degree of hypertrophy in the horizontal axis gradually increases from the lower epidermal cells in the feeding zone upto the upper epidermal cells. Structural diversity is poorer in *Pothos* galls than in the other galls, except the vertical divisioning of the palisade primordial cells in the young galls, hypertrophied cells and also the inhibition of the development of air spaces in the spongy mesophyll cell layers. There is no abnormal vascular differentiation in these galls. They show numerous feeding spots on the surface (plates 5A–C) owing to continuous feeding and the well developed galls generally show depigmented spots due to extensive chlorosis.

Partial gall development is also seen in *Pothos scandens*, involving the rolling of only one half of the lamina. Galling involves initial anticlinal divisions in the palisade primordial cells followed by cellular hypertrophy of the epidermal and palisade mesophyll cells. Lateral as well as horizontal stretching of the cells is evident in *Pothos* galls (table 4). Nuclear volume increases by 2.54 times in gall tissues (table 4), while hyperplasy in terms of the addition of cell layers is absent. Table 4 shows the tissue reactions and the degree of hypertrophy in the nuclei of young, mature and old galls compared with normal tissues.

(*d*) *Vitis lanceolaria*

Liothrips viticola induces hypophyllous roll galls of *Vitis lanceolaria* involving the

inrolling of the laminar margins towards the midrib with the lower epidermal portion lining the gall chamber, thereby forming tubular galls on either side of the midrib throughout the length of the leaflets (plate 5E). All the five leaflets of the compound leaf are generally involved in galling.

The initial feeding injury caused by *Liothrips viticola* on the abaxial surfaces of the young, differentiating leaves of *Vitis* damages 1–2 epidermal cells and hypodermal cells, which get necrosed (plate 8F). Simultaneously the primordial spongy mesophyll cells exhibit meristematic activity. The lower epidermis and a few layers of the spongy mesophyll cells show repeated periclinal divisions and as a result of the subcellular differentiation in the epidermal and the succeeding layers of cells, a cytologically specialised nutritive zone is formed with dense vacuolated cytoplasm and prominent nuclei. In mature galls, the normal dorsiventral differentiation of the leaves is completely absent and the bulk of the gall is constituted as a result of the repeated anticlinal and periclinal divisions of the mesophyll cells. The cells of the lower epidermis lining the gall chamber exhibit proliferation (Plate 8B). Tanniniferous cells appear in the young galls, the maximum number being found in old galls (plates 8D & F). Vacuolation is evident in the tanniniferous cells as well (plate 8 D). The complete structural deformation of the midrib region due to excessive divisions of the parenchyma cells nearer the vascular bundles pushing them laterally appears striking (plates 8C & E). The phloem parenchyma cells of the vascular bundle show cellular hypertrophy (plate 8A), not evident in the other galls reported here. Irregular cutinisation of the lower epidermal cells in the feeding region (plate 8B) is another feature characteristic of the gall tissues. Cells bearing crystals found along the margins of the radiating phloem strands, a characteristic anatomical feature in the normal leaves, are not found in the gall tissues.

The increase in nuclear volume is highly significant in gall tissues (table 5). Hypertrophy involving the stretching of the cells in the horizontal axis is greatest in mature and old galls, while lateral stretching is also evident in young, mature, and old gall tissues (table 5). Hypertrophy in the horizontal plane restricted to cells away from the nutritive zone, whereas lateral stretching is seen only in nutritive cells.

Histochemistry of the Normal and Galled Tissues

Histochemical studies on gall tissues are of recent origin, the existing information being confined to galls induced by acarines, homopterans, dipterans, and hymenopterans. Extensive investigations by Bronner (1969–1980) provide valuable information on the histochemistry of various gall tissues induced by cynipids, cecidomyiids, and nematodes discussing the localisation and the possible functions of polysaccharides, aminoacids, soluble amines, invertase, amylase, glucose-6-phosphatase, acid phosphatase, lipids, and starch in these abnormal growth systems. An attempt has been made here to examine the histochemical picture of the gall tissues of *Calycopteris floribundus*, *Loranthus elasticus*, *Pothos scandens*, and *Vitis lanceolaria*, involving the localisation of acid phosphatase, peroxidase, polyphenol oxidase, proteins, lipids, starch, condensed tannin precursors, and tannins through their phases of growth.

(*a*) *Acid phosphatase*

In *C. floribundus*, the enzyme acid phosphatase is localised in the palisade mesophyll cells, phloem parenchyma cells and also in the upper and lower epidermal cells of the young leaf primordia (plate 9G). The concentration of this enzyme in mature and old leaves was low, found only in the upper

Table 4 *Cellular hypertrophy, hyperplasy and nuclear hypertrophy in* Pothos scandens

Age of the normal and galled leaves		Cellular hypertrophy				Cellular hyperplasy		Nuclear hypertrophy	
		Lateral streching		Horizontal stretching		Number of cell layers		No. of times increase in nuclear volume in gall when compared to normal ones	
		Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value
3—6 days	Young normal leaf	23.43±0.20 (18.56—25.52)	9.58*	15.54±0.26 (11.6—20.88)	2.82*	5.5±0.01 (5—6)	—		4.25*
	Young gall	48.49±0.74 (39.44—64.96)		20.88±0.52 (16.24—30.16)		5.5±0.01 (5—6)		1.57±0.04 (1.11—2.23)	
12—16 days	Mature normal leaf	25.98±0.32 (23.2—32.48)	3.22*	19.00±0.38 (13.92—20.88)	4.19*	6.5± 0.01 (6—7)	—		8.58*
	Mature gall	35.26±0.95 (25.52—53.36)		29.92±0.74 (25.52—39.44)		6.1±0.01 (6—7)		2.94±0.07 (1.82—4.16)	
20—23 days	Old normal leaf	22.92±0.39 (20.88±30.16)	6.11*	20.65±0.46 (16.24—25.52)	2.80*	7.5±0.01 (7—8)	—		5.96*
	Old gall	29.46±0.54 (25.52—39.44)		48.02±0.12 (34.8—69.6)		7.5±0.01 (7—8)		3.16±0.12 (1.69—5.85)	

*Significant at 1% level = 2.552
Values in the parenthesis indicate range.

Table 5 *Cellular hypertrophy, hyperplasy and nuclear hypertrophy in* Vitis lanceolaria

Age of the normal and galled leaves		Cellular hypertrophy				Cellular hyperplasy		Nuclear hypertrophy	
		Lateral stretching		Horizontal stretching		Number of cell layers		No. of times increase in nuclear volume in gall when compared to normal ones	
		Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value	Mean ± S.E	*t*. test value
	Young normal	19.02 ± 0.34 (13.92—23.2)		15.08 ± 0.36 (11.6—20.88)		7.5 ± 0.01 (7—8)			
2—5 days	leaf		3.18*		4.12*		3.12*		5.05*
	Young gall	30.16 ± 1.03 (23.2—46.4)		32.02 ± 1.18 (18.56—53.36)		14.0 ± 0.12 (12—16)		1.82 ± 0.05 (1.12—2.67)	
	Mature normal	29.00 ± 0.69 (16.24—34.8)		18.10 ± 0.78 (11.6—34.8))		9.0 ± 0.14 (8—10)			
10—15 days	leaf		4.90*		6.05*		4.10*		8.82*
	Mature gall	40.37 ± 0.81 (27.84—44.08)		47.33 ± 0.89 (32.48—62.64)		23.0 ± 0.23 (18—26)		2.61 ± 0.08 (1.43—3.86)	
	Old normal	27.38 ± 0.29 (27.84—34.8)		17.40 ± 0.40 (13.92—25.52)		9.5 ± 0.02 (10—11)			
20—25 days	leaf		6.65*		8.60*		4.32*		8.14*
	Old gall	49.65 ± 0.96 (37.12—64.96)		57.96 ± 1.36 (37.12—69.6)		29.0 ± 0.31 (25—32)		4.34 ± 0.17 (2.57—7.43)	

*Significant at 1% level = 2.552
Values in the parenthesis indicate range.

epidermis, palisade cells, and phloem cells (plate 9H). Although a high content of acid phosphatase was evident in the inner and outer epidermises, nutritive tissue, and phloem parenchyma cells of the young, mature, and old galls, its wall-bound nature in xylary elements and some of the parenchyma cells with the maximum being in nutritive tissues of young galls (plates 9A–F) was interesting. The normal leaf tissues of all the stages of *Loranthus* exhibited low stain reactions for acid phosphatase in the epidermal and phloem cells, and in mature and old leaves in palisade cells. Gall tissues of *Loranthus* showed a random distribution of acid phosphatase throughout the mesophyll tissues, the maximum being in nutritive tissues in younger stages except in the xylary elements. The phloem elements showed a considerably low profile.

Less activity of this enzyme was evident in the upper epidermis and vascular tissues in young leaves of *Pothos*, whereas in mature leaves it was restricted to the phloem cells and palisade mesophyll cells and totally absent in old leaves. At the same time the gall tissues of *Pothos* exhibited a higher staining reaction for this enzyme in the young gall tissues occurring mostly in lower epidermal cells and nutritive tissues, peripheral parenchyma cells, and phloem parenchyma cells. In mature galls it was found to be maximum in the nutritive zone and its vicinity whereas the old gall tissues exhibited a negative reaction.

Among the three different stages of the normal leaves of *Vitis*, the maximum activity of this enzyme was recognised in the spongy mesophyll cells phloem and epidermal cells of young leaves, while it was totally absent in old leaves. As observed in other galls, this enzyme activity is maximum in young and mature galls, being accumulated mostly in nutritive tissues, vascular tissues, and epidermal cells. Apart from this it occurs in the medullary ray cells and wall-bound in xylary elements of normal as well as gall tissues.

(*b*) *Peroxidase*

Peroxidase exhibited positive reactions in young and mature leaf tissues of *Calycopteris* confined to upper epidermis, palisade initials, and phloem cells (plates 10 A & F), showing negative reactions in old leaves. Strong enzyme activity was noticed in young, mature, and old galls, being maximally localised in nutritive tissues, upper and lower epidermal cells, phloem parenchyma, and xylem vessels (plates 10B, C, D, G, & H). Peroxidase also occurred in the parenchyma cells of the nutritive zone being bound to the cell walls (plate 10 E). A high intensity of stain reactions for peroxidase was found throughout the gall tissues of *Loranthus*, especially in phloem parenchyma, epidermal cells, and nutritive cells of young and mature galls. In old galls it was localised mostly in the epidermal and parenchyma cells away from the nutritive tissues. Peroxidase was accumulated in isolated epidermal cells and phloem parenchyma cells in all the three stages of normal leaves of *Loranthus*. Gall tissues as a whole exhibited strong reactions of peroxidase in young, mature, and old stages except in xylem. Wall-bound activity of this enzyme was evident in epidermal cells, collenchyma and parenchyma cells of the midrib region. Nutritive tissues of old galls did not show any significant results for the histochemical reactions of this enzyme. A random distribution of this enzyme was evident in all the cells of the gall tissues of *Pothos*. Negative reaction for peroxidase was observed in the peripheral cells of the old gall tissues. Localised reactions were seen in the phloem, spongy and palisade parenchyma cells of young and mature leaves of *P. scandens*. Old leaf tissues showed a considerably low profile of this enzyme restricted to epidermal cells. In the healthy

leaf tissues of *Vitis*, peroxidase activity was recognised in all the tissues of mature leaves except in xylem elements, whereas young and old leaves exhibited its presence in the epidermises spongy mesophyll cells and phloem parenchyma cells (plates 11 A & F). Among the gall tissues of *Vitis*, phloem cells, nutritive tissues and epidermal cells appeared rich in peroxidase activity (plates 11 B & E).

(c) Polyphenol oxidase (PPO)

In young and mature leaves PPO occurred in a few cells of palisade and spongy mesophyll cells and also in the phloem, lower and upper epidermal cells (plates 12 G, H & I). PPO activity was found in gall tissues localised maximally in young galls, concentrated in the nutritive tissues and gall peripheral cells (plates 12A–C). As the gall became old, a gradual decrease in concentration of this enzyme was evident. PPO was present in the nutritive zone, epidermal cells, and vascular tissues of young and mature galls, whereas in old galls a considerably low profile was observed in cells away from the nutritive zone and also in vascular tissues (plates 12 D–F).

Polyphenol oxidase was found throughout the gall tissues of *Loranthus* in all the three stages and the maximum concentration was seen in nutritive tissues and also in cells away from them. In young, mature, and old leaves of *L. elasticus*, localisation of this enzyme has been observed in both the epidermal cells, palisade and phloem parenchyma cells in low concentrations. Normal leaf as well as gall tissues of *Pothos* exhibited considerably reduced reactions for PPO. The enzyme was located in the epidermal cells and peripheral gall tissues irrespective of the age of the gall (plates 13A, B & C). In the normal tissues it was noticed in the palisade parenchyma cells and epidermal cells (plate 13 D), but was totally absent in old leaves.

PPO was present only in young and mature leaf tissues of *Vitis lanceolaria*, confined to epidermal, mesophyll, and phloem parenchyma cells of young leaves and in palisade and spongy mesophyll tissues of mature leaf being totally absent in old leaf tissues. In gall tissues it got accummulated in the inner epidermal cells, nutritive tissues, and phloem parenchyma cells in all the three stages of development.

(d) Proteins

Gall tissues of *Calycopteris* exhibited a maximum concentration of protein in the cells of the nutritive zone and epidermal cells lining the nutritive zone with a considerable amount in pholem parenchyma cells also (plates 14A, B, D & E). In the normal tissues it was totally absent in old leaves while occurring in phloem parenchyma, medullary ray cells, palisade and spongy mesophyll cells of young and mature leaves (plates 14 C & F). A higher concentration of proteins was evident in the gall tissues of *Loranthus* being distributed maximally in the nutritive tissues, epidermal cells lining them, and vascular tissues irrespective of the age of the gall (plates 15A–C & E). Palisade cells, epidermises and phloem parenchyma cells of all the stages of normal leaves were also rich in protein (plate 15D). In gall tissues of *Pothos* proteins exhibited a high stain reaction in phloem parenchyma cells, nutritive tissues and also in cells in their vicinity (plates 16A–D & F). In all the three stages of normal leaves, protein occurred in the palisade and spongy mesophyll cells (plate 16G). Healthy old leaves of *P. scandens* showed a considerably low profile of protein in phloem as well as in lower epidermal cells apart from mesophyll cells (plate 16 E). *Vitis* galls exhibited protein concentrations in nutritive tissues, inner epidermal cells, and phloem parenchyma cells of galls irrespective of the stage of development whereas in normal leaf tissues protein appeared in lower epidermis, spongy

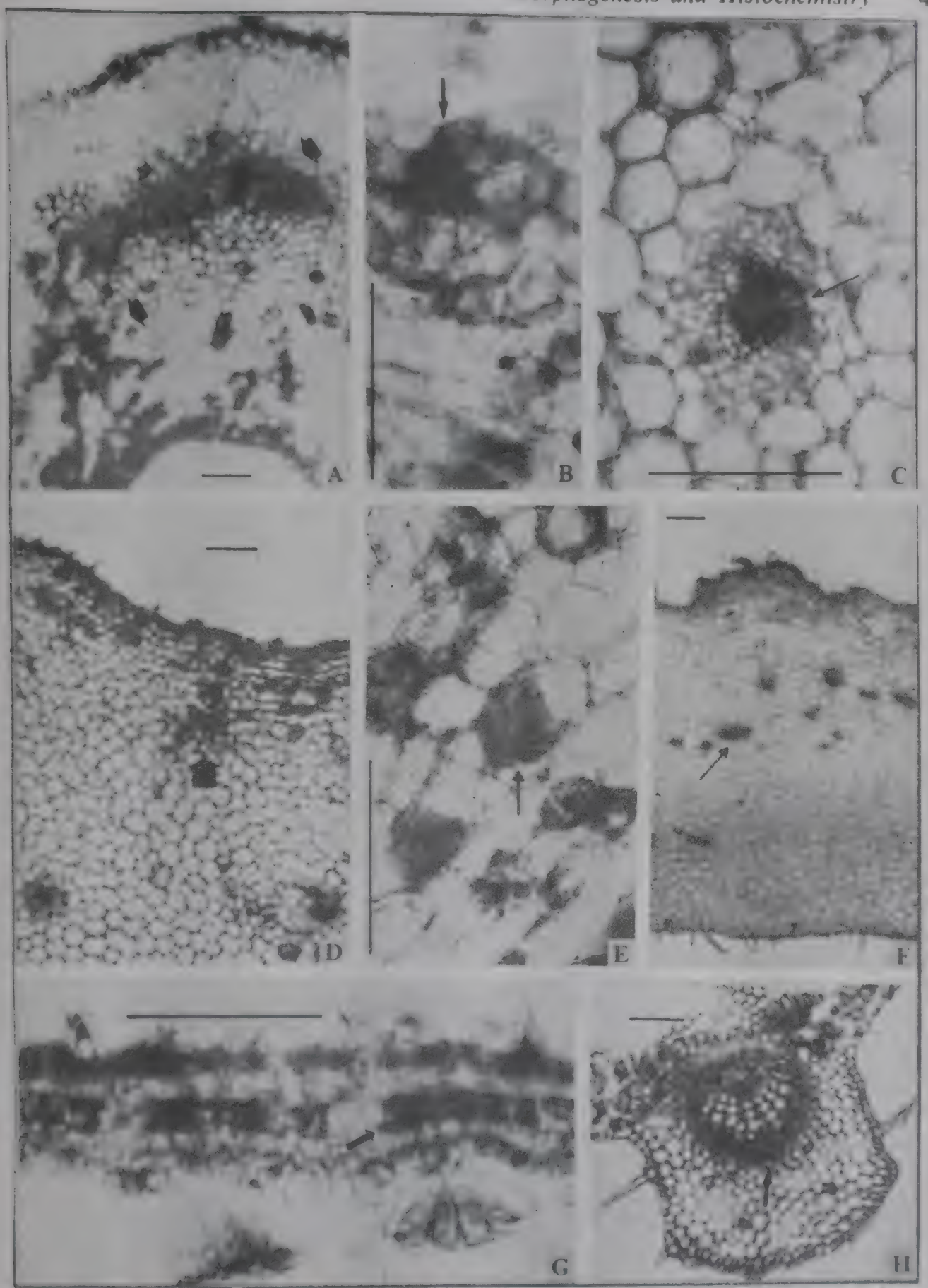

Plate 9 *Calycopteris floribundus*. (A) Young gall (TS)—Acid phosphatase activity in nutritive tissues and vascular tissues; (B) Young gall (TS)—Acid phosphatase activity in nutritive zone; (C) Mature gall (TS)—Acid phosphatase activity in vascular tissues; (D) Mature gall (TS)—Acid phosphates activity in cells away from the nutritive zone and vascular tissues; (E) Young gall (TS)—Acid phosphatase activity in nutritive cells; (F) Old gall (TS)—Acid phosphatase activity in nutritive zone; (G) Young leaf (TS)—Acid phosphatase activity in epidermal and primordial mesophyll cells; (H) Mature leaf (TS)—Acid phosphatase activity in phloem and wall bound in collenchyma cells

Scale: Line represents 100 μm.

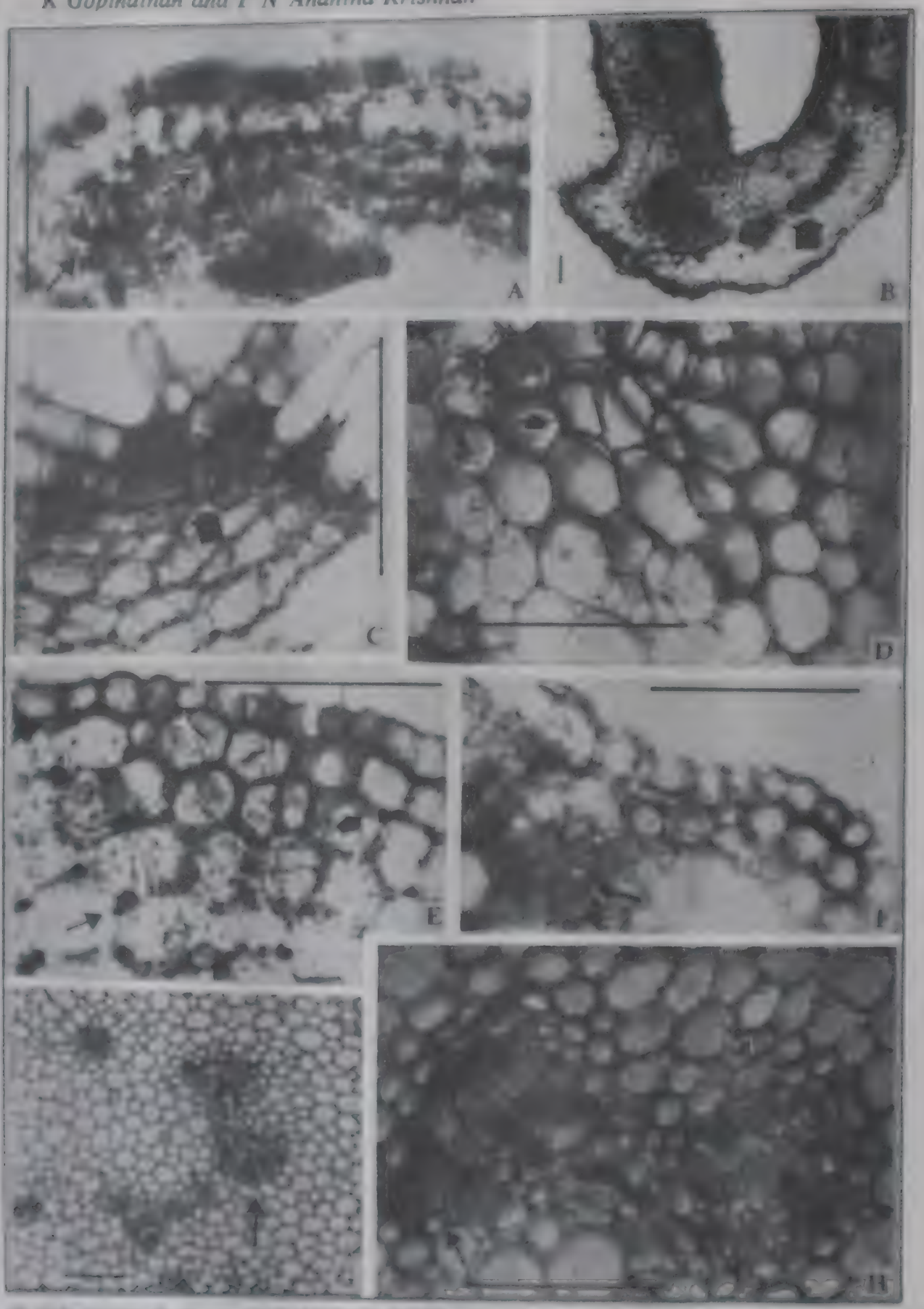

Plate 10 *Calycopteris floribunda*. (A) Young leaf (TS)—Peroxidase activity in primordial palisade mesophyll cells and epidermis; (B) Young gall (TS)—Peroxidase activity in nutritive zone, epidermises and vascular tissues; (C) Young gall (TS)—Peroxidase activity in nutritive zone. (D and E) Mature gall (TS)—Peroxidase activity in nutritive cells and in cells away from them respectively. (F) Mature leaf (TS)—Peroxidase activity in epidermis and palisade cells; (G and H) Old gall (TS)—Peroxidase activity in vascular tissues and wall bound in xylary elements

Scale: Line represents 100 μm

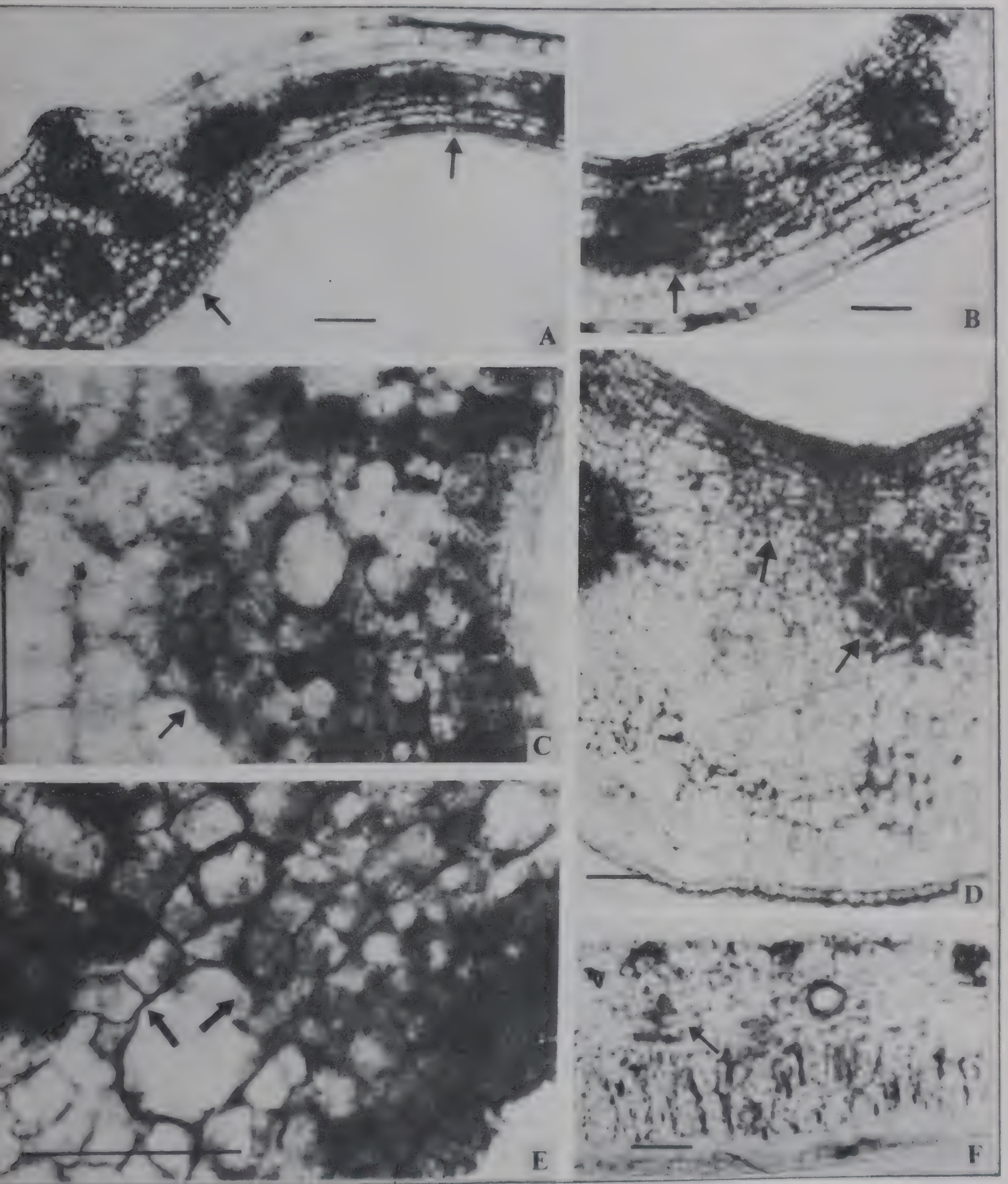

Plate 11 *Vitis lanceolaria*. (A) Young leaf (TS)—Peroxidase activity in epidermises, spongy and phloem parenchyma cells; (B) Young gall (TS)—Peroxidase activity in epidermis, nutritive tissues and phloem cells; (C) Young gall (TS)—Peroxidase activity in nutritive tissues; (D and E) Mature gall (TS)—Peroxidase activity in nutritive tissues and epidermises; (F) Old leaf (TS)—Peroxidase activity in epidermal cells
Scale: Line represents 100 μm

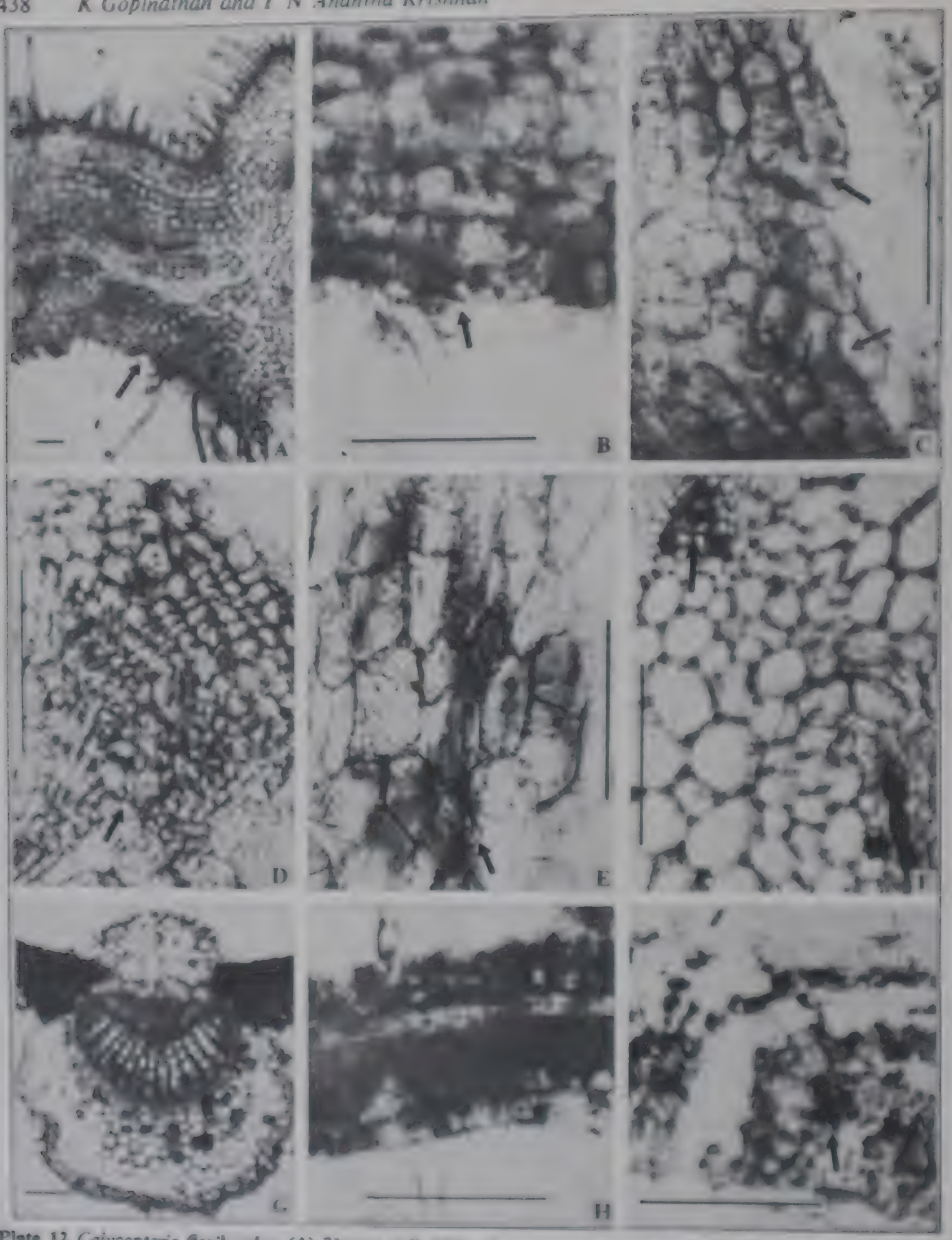

Plate 12 *Calycopteris floribunda*. (A) Young gall (TS)—Polyphenoloxidase activity in nutritive cells, cells farther away and epidermal cells; (B) Young gall (TS)—Polyphenoloxidase activity in nutritive cells; (C) Young gall (TS)—Polyphenoloxidase activity in cells away from the nutritive zone. (D and F) Mature gall (TS)—Polyphenoloxidase activity in phloem parenchyma and wall bound in xylary elements. (E) Mature gall (TS)—Polyphenoloxidase activity in nutritive cells. (G) Mature leaf (TS)—Polyphenoloxidase activity in phloem and parenchyma cells of the mid-rib region. (H) Young leaf (TS)—Polyphenoloxidase activity in epidermal cells and mesophyll cells; (I) Mature gall (TS)—Polyphenoloxidase activity in palisade and spongy mesophyll cells. *Scale*: Line represents 100 μm

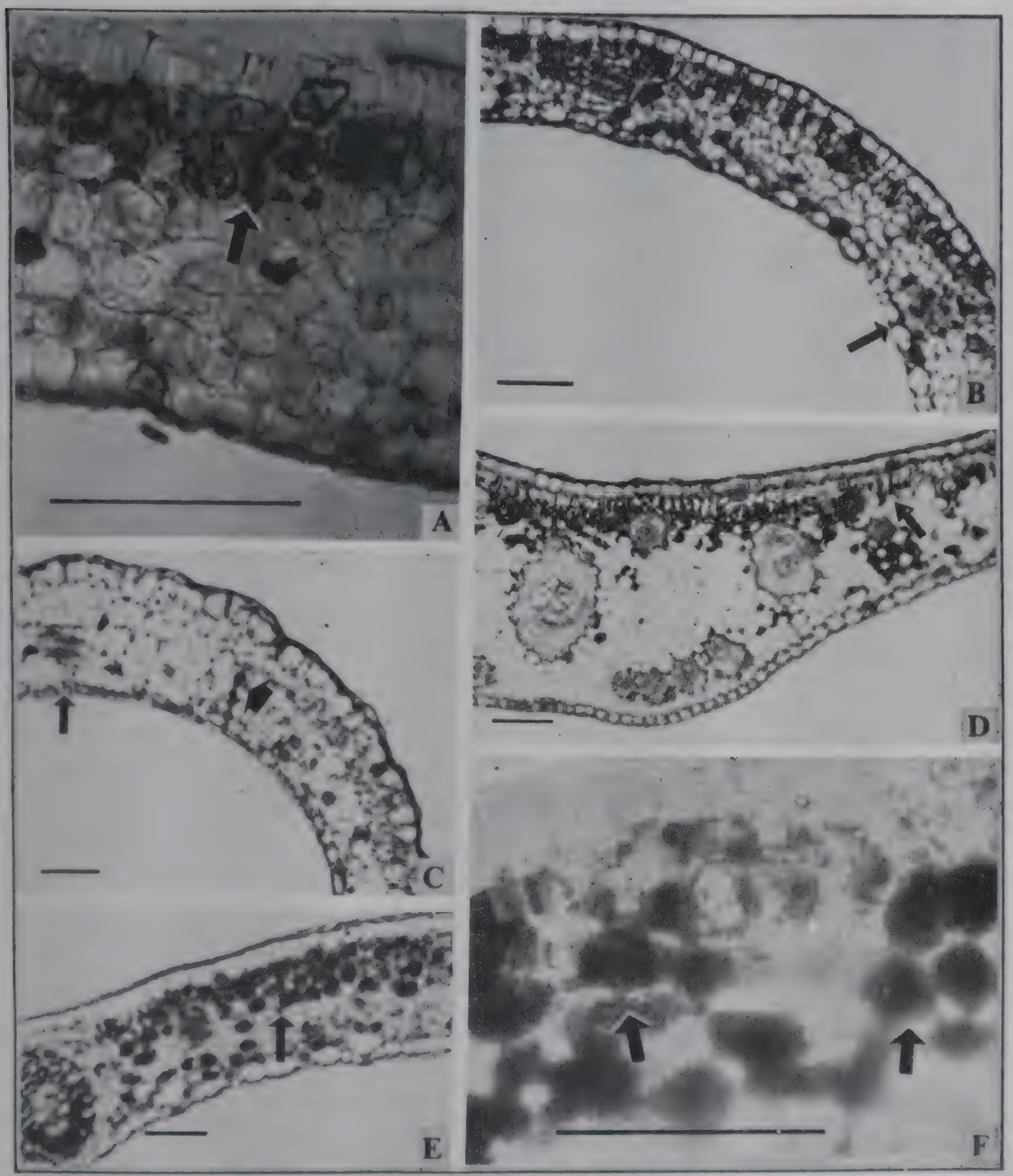

Plate 13 *Pothos scandens.* (A) Young gall (TS)—Polyphenoloxidase activity in cells away from the feeding site and epidermal cells; (B) Mature gall (TS) Polyphenoloxidase activity in epidermises and cells away from the feeding site; (C) Old gall (TS)—Polyphenoloxidase activity in epidermal cells and cells away from the feeding site; (D) Mature leaf (TS)—Polyphenoloxidase activity in palisade parenchyma and epidermal cells; (E) Mature gall (TS)—Starch deposits in cells away from the feeding site; (F) Old gall (TS)—Starch deposits in cells away from the feeding site

Scale: Line represents 100 μm

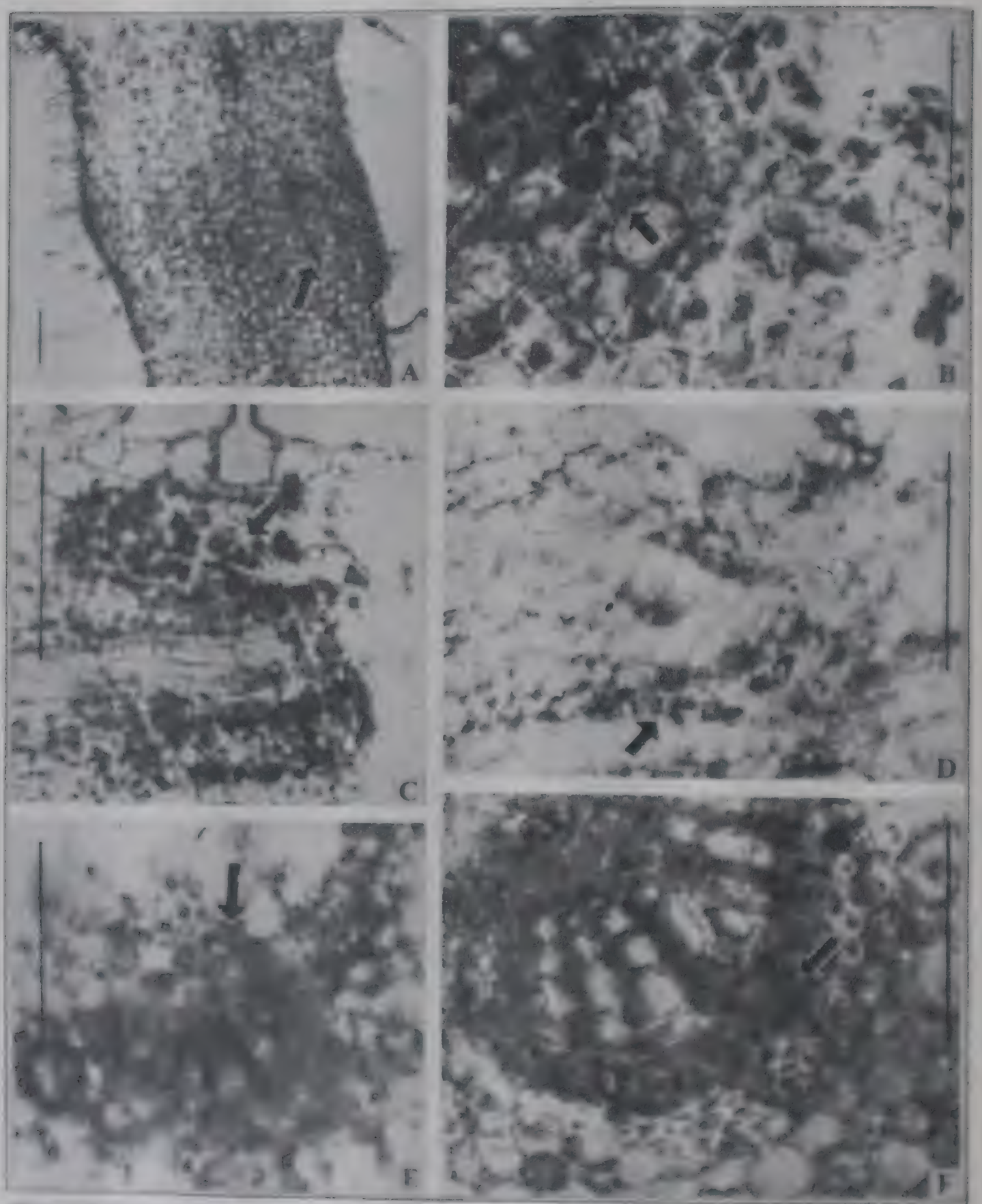

Plate 14 *Calycopteris floribundus*. (A and B) Young gall (TS)—Protein localised in nutritive tissues; (C) Mature leaf (TS)—Protein localised in palisade and spongy mesophyll tissues, (D) Old gall (TS)—Protein localised in nutritive and vascular tissues; (E) Mature gall (TS)—Protein localised in nutritive tissues; (F) Mature leaf (TS)—Protein localised in the medullary ray cells and phloem parenchyma cells of the midrib region.
Scale: Line represents 100 μm

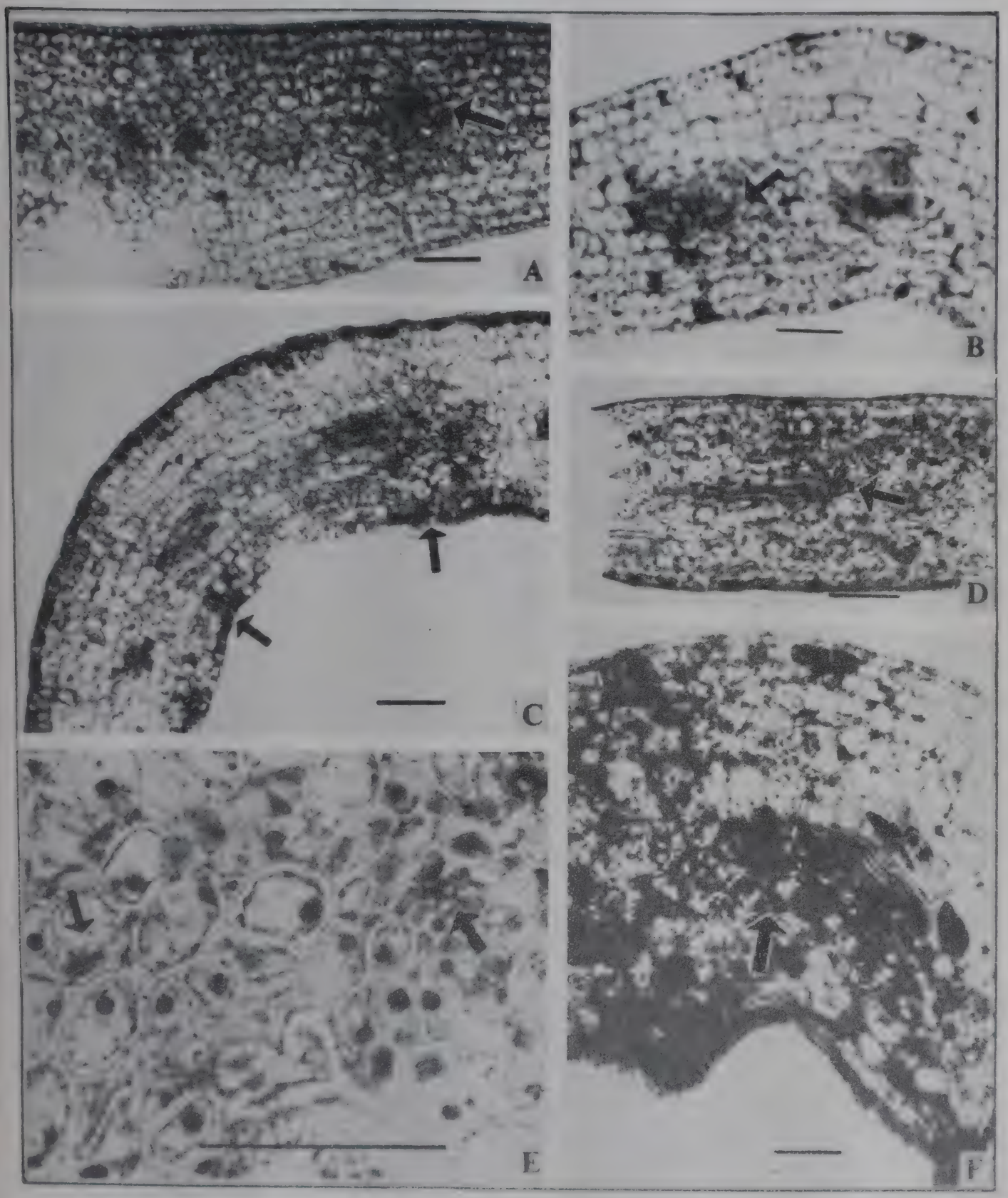

Plate 15 *Loranthus elasticus*. **(A)** Young gall (TS)—Protein localised in nutritive and epidermal cells; **(B)** Old gall (TS)—Protein localised in nutritive tissues and phloem parenchyma cells; **(C)** Mature gall (TS)—Protein localised in nutritive tissues, vascular tissues and epidermal cells; **(D)** Mature leaf (TS)—Protein localised in palisade cells, epidermises and phloem parenchyma cells; **(E)** Young gall (TS)—Protein localised in nutritive tissues; **(F)** Mature gall (TS)—Starch deposits in nutritive tissues and cells away from them
Scale: Line represents 100 μm

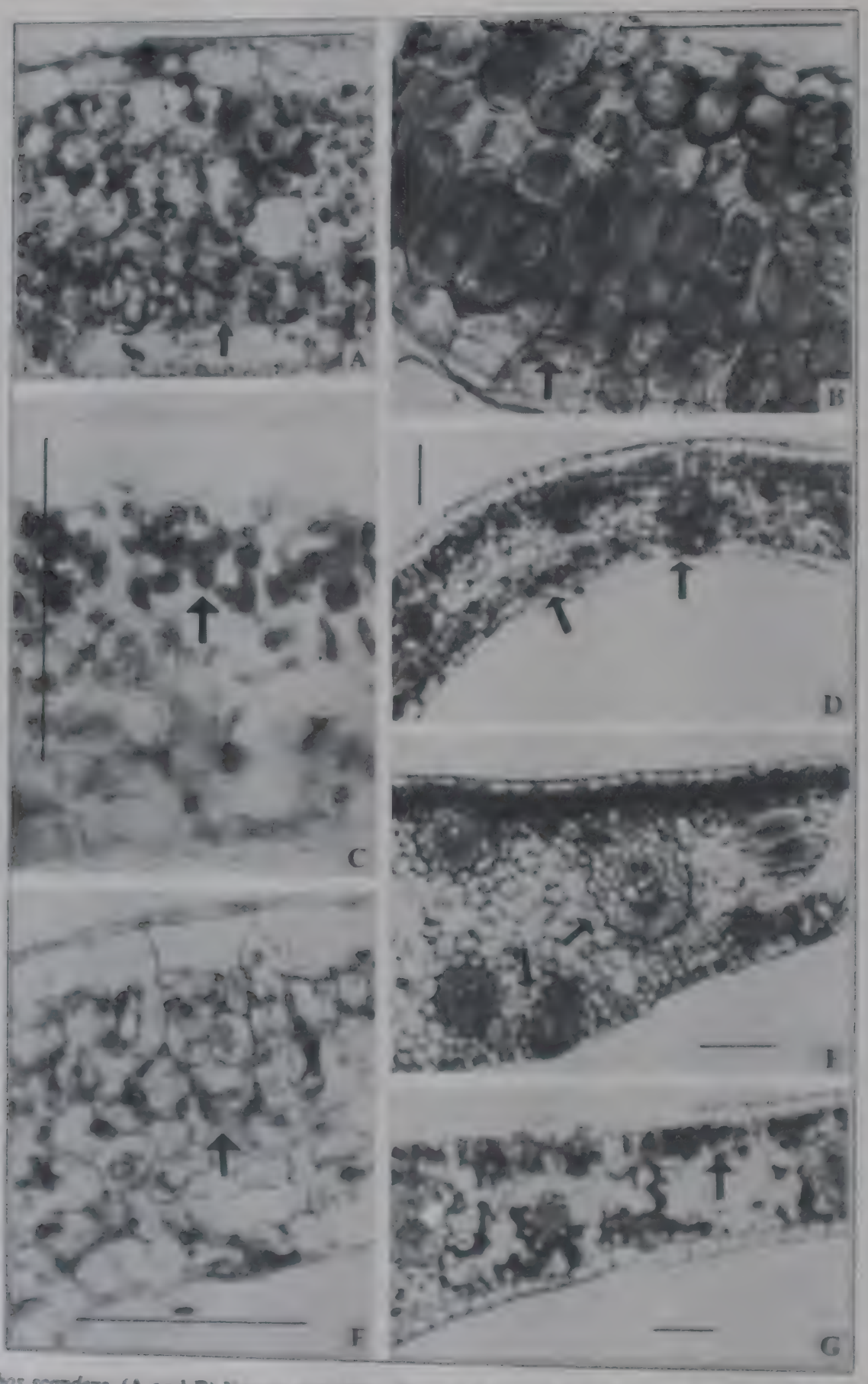

Plate 16 *Pothos scandens*. (A and B) Young gall (TS)—Protein localised in cells near the feeding site and farther away; (C and D) Mature gall (TS)—Protein localised in cells near the feeding site and away from it; (E) Old leaf (TS)—Protein localised in isolated mesophyll cells, phloem and epidermal cells; (F) Old gall (TS)—Protein localised in cells away from the feeding site; (G) Mature leaf (TS)—Protein localised in mesophyll tissues *Scale*: Line represents 100 μm

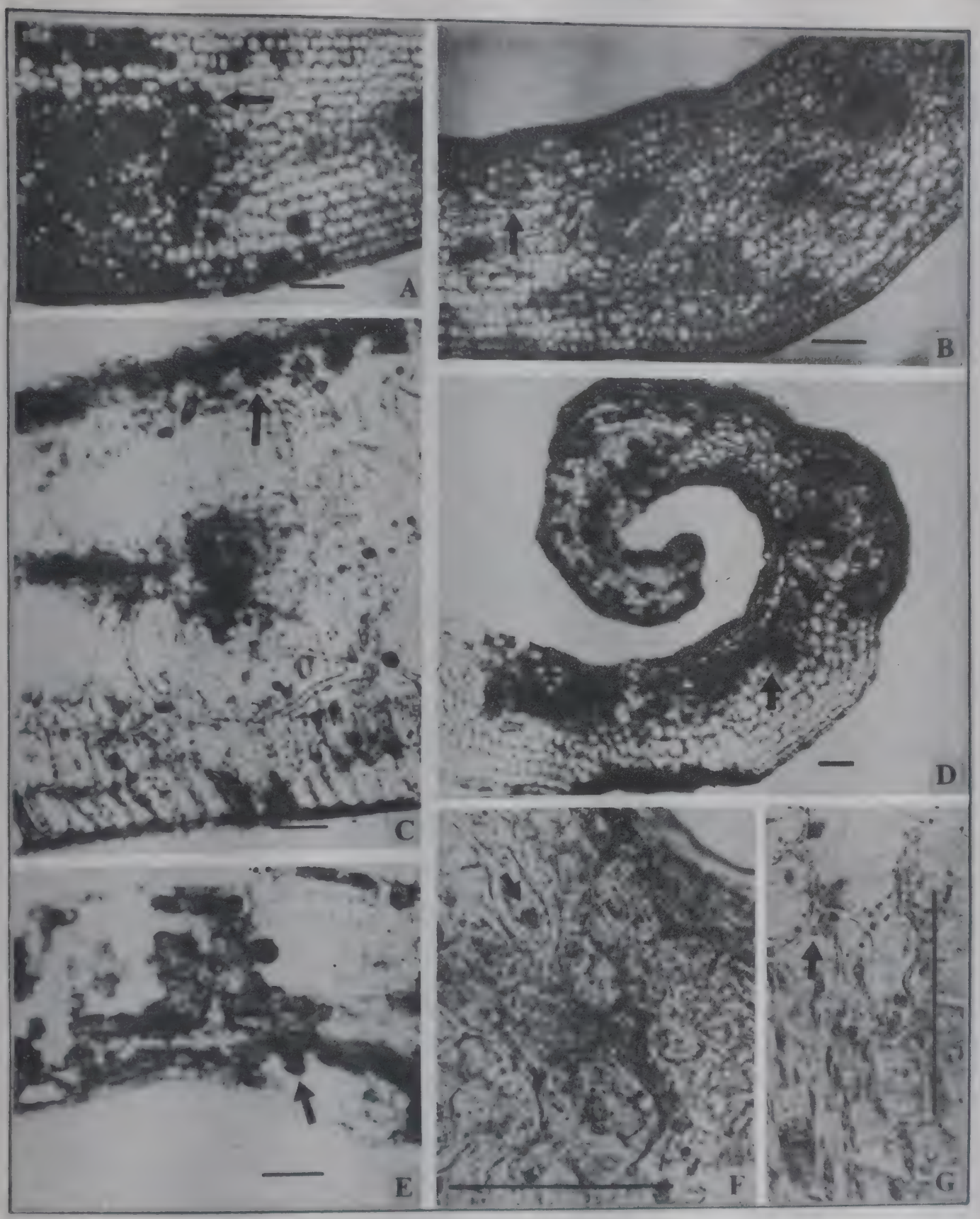

Plate 17 *Loranthus elasticus*. (A) Young leaf (TS)—Tannin localised in epidermises, parenchyma cells of the midrib and phloem cells; (B) Young gall (TS)—Tannin localised in nutritive tissues and cells away from them; (C) Mature leaf (TS)—Tannin localised in epidermal and palisade mesophyll cells; (D) Old gall (TS)—Tannin localised throughout the gall tissues; (E) Mature gall (TS)—Tannin localised in nutritive tissues and cells away from them; (F) Old gall (TS)—Lipid droplets in nutritive tissues and epidermal cells; (G) Mature gall (TS)—Lipid droplets in the cells away from the nutritive tissues

Scale: Line represents 100 μm

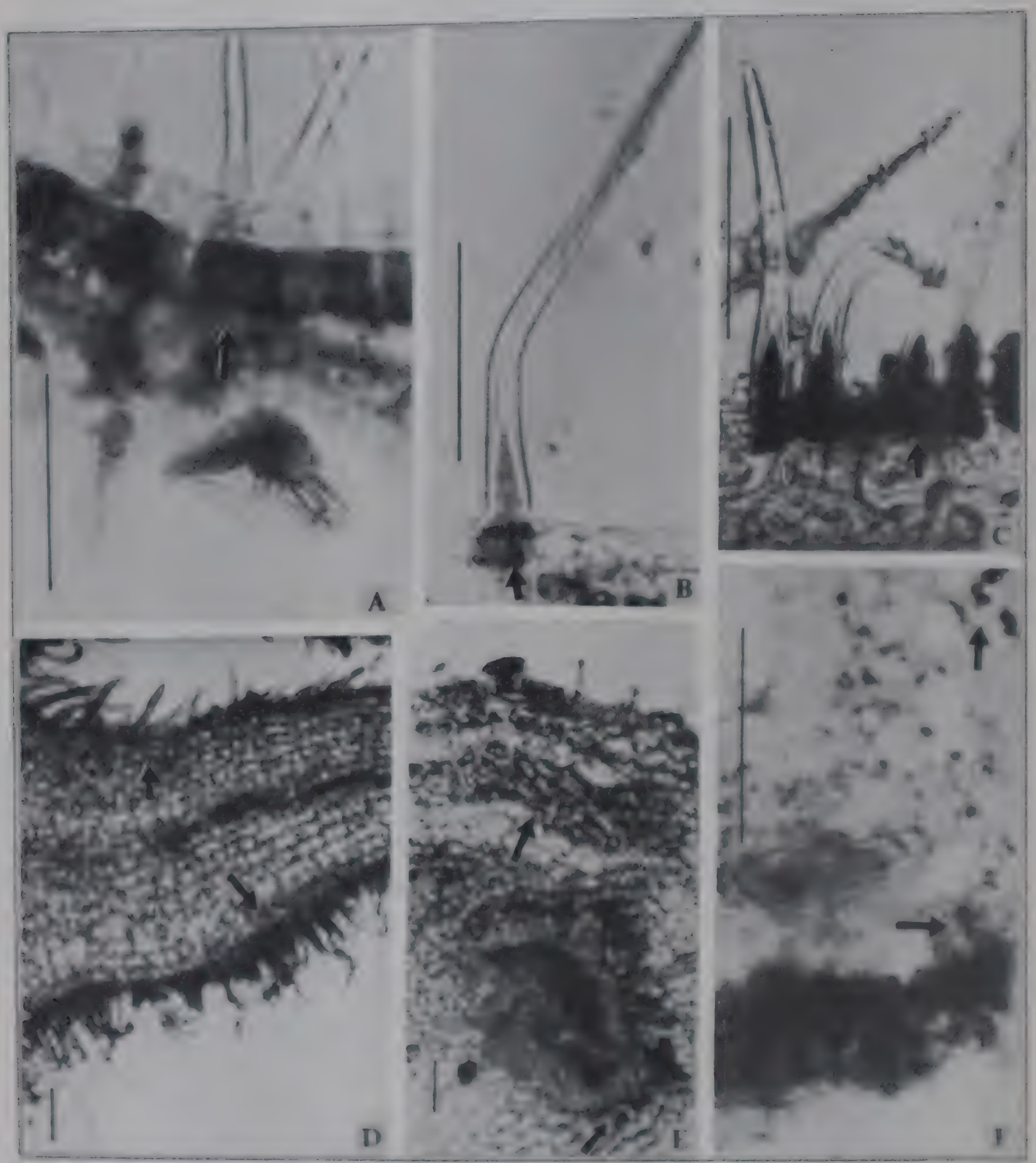

Plate 18 *Calycopteris floribunda*. (A) Young leaf (TS)—Condensed tannin precursors localised in primordial mesophyll cells. (B) Young leaf (TS)—Condensed tannin precursors localised in the epidermis and unicellular hairs. (C) Mature leaf (TS)—Condensed tannin precursors localised in unicellular hairs. (D) Mature gall (TS)—Condensed tannin precursors localised in trichomes, epidermises, vascular tissues and parenchyma cells in the nutritive zone. (E) Old gall (TS)—Starch deposits in the outer epidermis and cells away from the nutritive zone. (F) Mature leaf (TS)—Starch deposits in spongy mesophyl cells and parenchyma cells of the midrib region. *Scale:* Line represents 100 μm

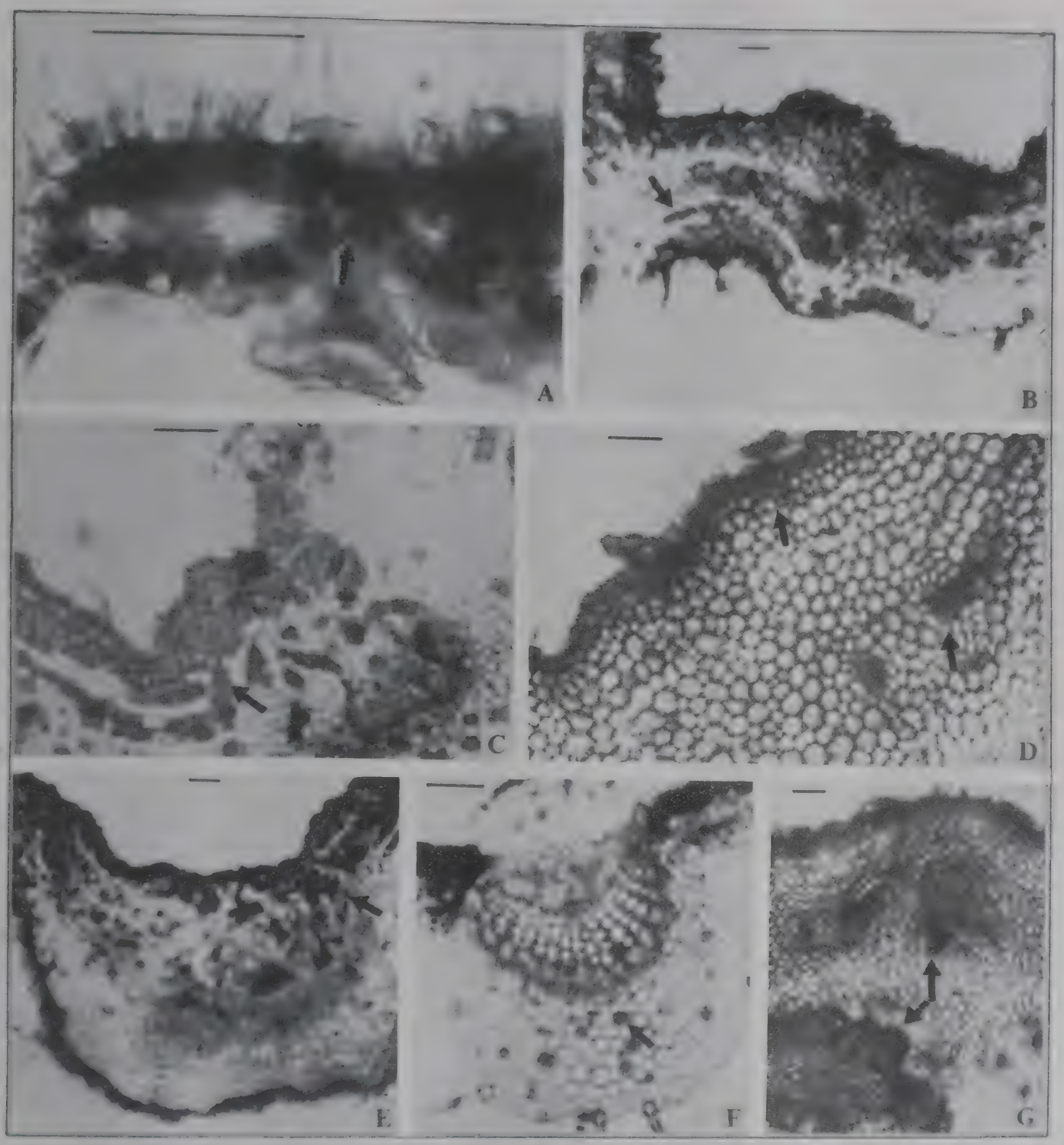

Plate 19 *Calycopteris floribundus*. **(A)** Young leaf tissues (TS)—Tannin localised in palisade and spongy mesophyll initials; **(B)** Young gall tissues (TS)—Tannin localised in the nutritive tissues and other gall tissues. **(C)** Mature gall (TS)—Tannin localised in the nutritive tissue; **(D)** Mature gall (TS)—Tannin localised in outer epidermal cells and cells away from the nutritive tissues; **(E)** Old gall (TS)—Tannin localised in the nutritive tissues; **(F)** Mature leaf (TS)—Tannin localised in the parenchyma cells of the midrib region; **(G)** Old gall (TS)—Tannin localised in nutritive cells and cells away from them

Scale: Line represents 100 μm

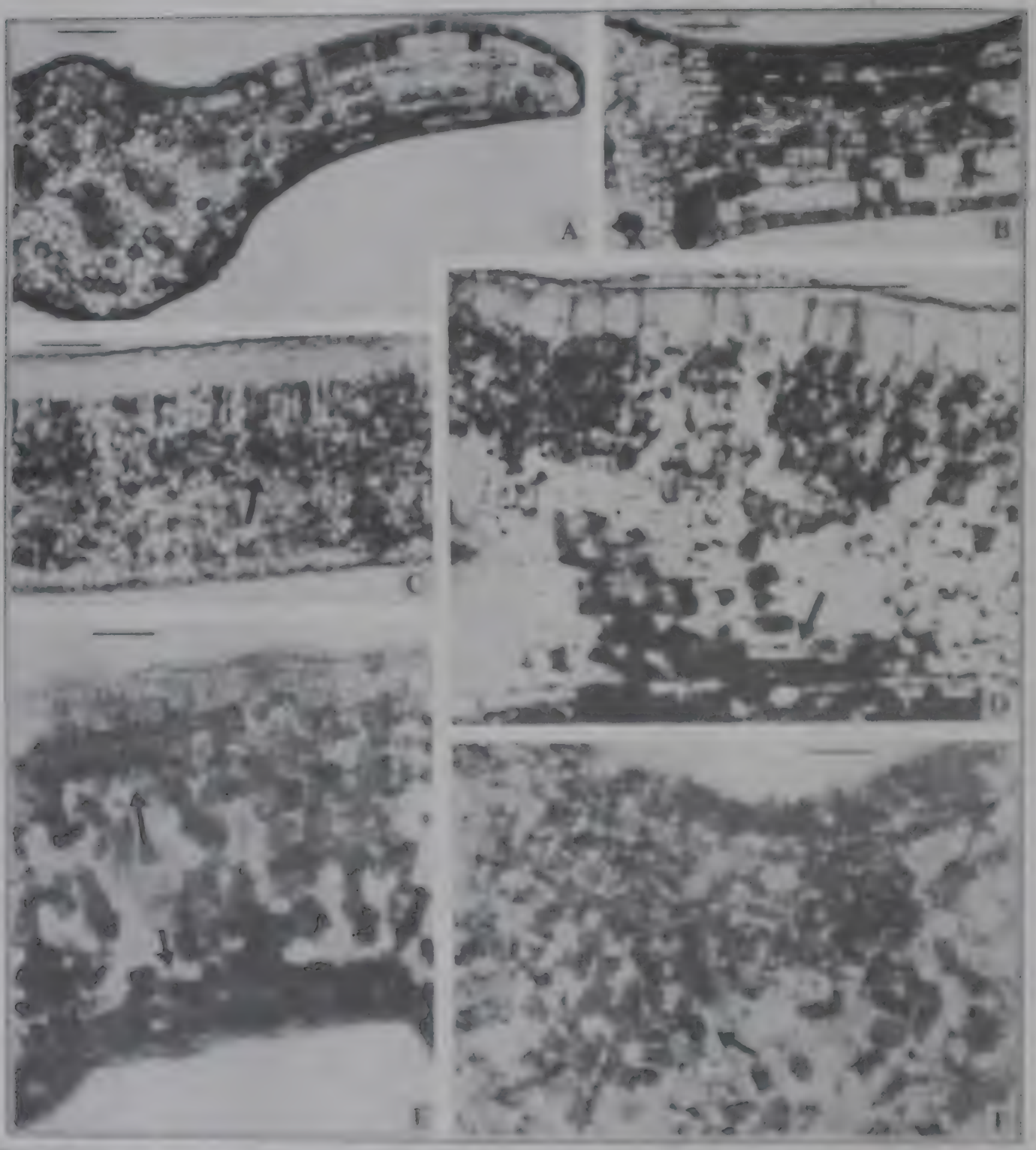

Plate 20 *Vitis lanceolaria*; (A) Young leaf (TS)—Tannin localised in primordial mesophyll initials and epidermal cells. (B) Young gall (TS)—Tannin localised in epidermal cells and nutritive tissues. (C) Mature leaf (TS)—Tannin localised in epidermal cells and palisade mesophyll tissues. (D) Old leaf (TS)—Tannin localised in palisade and spongy mesophyll tissues; (E) Mature gall (TS)—Tannin localised in nutritive and other tissues. (F) Old gall (TS)—Tannin localised in nutritive tissues and epidermal cells lining them.
Scale: Line represents 100 μm

mesophyll cells, and phloem tissues in young leaves. The concentration of protein decreased in mature and old leaves being restricted to phloem parenchyma cells.

(*e*) *Lipids*

Lipids were totally absent in normal and gall tisses of *Calycopteris*. In mature and old gall tissues of *Loranthus* lipid droplets were found accumulated in both the epidermal cells, nutritive tissues, and in the peripheral cells away from the nutritive tissues (plates 17F & G), while they were absent in young gall tissues and healthy leaf tissues. Gall tissues in general showed lipid droplets in the nutritive tissues and epidermal cells of *Pothos*, lipids exhibited a negative stain reaction. They were totally absent in normal as well as in gall tissues of *Vitis*.

(*f*) *Starch*

Starch grains were present in the spongy mesophyll cells and also in isolated parenchyma cells of the midrib region of the mature and old leaves of *Calycopteris* (plate 18F). In mature and old gall tissues of *Loranthus*, starch grains appeared abundant in nutritive zone cells, epidermal cells of the nutritive zone, and in the cells away from the nutritive zone (plate 15F). In the mature and old galls of *Pothos*, starch deposits were detected in nutritive zone cells and also in gall tissues away from the nutritive zone (plates 13 E & F). Starch deposits appeared rich in spongy and palisade mesophyll cells of old leaves and randomly distributed in the mature and old gall tissues of *Vitis*.

(*g*) *Condensed Tannin Precursors*

Condensed tannin precursors were seen in considerable concentrations in both the epidermal cells and palisade mesophyll initials of young leaf primordia of *C. floribundus* and in the epidermal hairs of leaves irrespective of the age of the leaves (plates 18A, B & C). Gall tissues of *Calycopteris* generally showed a higher concentration of condensed tannin precursors confined to epidermal cells and trichomes, with low stain reactions in the vascular tissues and isolated parenchyma cells irrespective of the age of the gall (plate 18D). Histochemical reactions for localization of condensed tannin precursors were negative in normal as well as in the gall tissues of *Loranthus* and *Pothos*.

Condensed tannins precursor activity was observed in the nutritive tissues and inner epidermal cells of young and mature galls of *Vitis*, whereas in old galls it was seen in peripheral parenchyma cells away from nutritive zone and phloem cells. In young and mature healthy tissues it was found in the epidermal cells, spongy parenchyma cells, and also in phloem parenchyma cells. Old leaf tissues exhibited a negative reaction.

(*h*) *Tannins*

Tanniniferous cells were evident in young, mature, and old stages of the leaves and gall tissues of *Calycopteris*, the maximum being in gall tissues. Palisade and spongy mesophyll cells of young, mature, and old leaves showed positive reactions to tannin (plates 19A & F) whereas higher concentrations of tannin were seen throughout the gall tissues except in vascular tissues and epidermal hairs (plates 19B–E & G).

In young leaf tissues of *L. elasticus*, tannin was noticed in the epidermal and parenchyma cells of the midrib and the phloem cells, whereas in mature and old leaves it was seen in palisade mesophyll cells apart from epidermal cells (plates 17A & C). Tannins accumulated in gall tissues as a whole in all kinds of cells except in vascular tissues irrespective of the age of the gall (plates 17B, D & E).

Histochemical reactions for tannins in the normal and gall tissues of *Pothos scandens* were negative. Tannin was predominant in the gall tissues of *Vitis* being confined to the epidermal cells, nutritive tissues, and the

cells in its vicinity of mature and old gall tissues (plates 20 E & F). In young gall tissues it was seen in epidermal cells and nutritive tissues (plate 20B). Young leaves of *V. lanceolaria* exhibited a maximum concentration of tannin in them compared to the other stages of leaves in which tannin was found localised in both the epidermal cells, palisade and spongy parenchyma cells (plate 20A). In mature leaves its presence was evident only in epidermal cells and palisade parenchyma cells whereas in old leaves it was accumulated only in the palisade and isolated spongy mesophyll cells (plates 20 C & D).

Discussion

(*a*) Morphogenesis of Thrips Galls in Relation to Normal Histogenesis

The hypophyllous leaf roll galls of *Pothos scandens* appear to be the simplest of all the galls studied, lacking a well defined nutritive tissue, meristematic activity, tissue differentiation, and addition of cell layers. *Pothos scandens* galls, in spite of their hypophyllous laminar rolling exhibited a simpler and minimal organisational pattern as compared to the better differentiated thrips galls.

In the development of epiphyllous roll gall of *Loranthus elasticus*, as a result of the reaction to wounding the palisade cells remain small and isodiametric, while the cells of the spongy mesophyll initially show perpendicular elongation exhibiting lateral stretching with the maturation of the gall. An exactly opposite phenomenon was observed in hypophylous rolling of *Vitis lanceolaria*. Eventhough the rolling pattern is different in these two types of galls, the morphogenetic gradient or the reaction process involved during development by the different types of tissues such as palisade or spongy mesophyll cells is more or less identical depending on their specific developmental requirements and the site of action. In both the epiphyllous and hypophyllous galls of *Loranthus* and *Vitis* respectively, a profound thickening of the lamina involved has been observed, similar to the observations of Karny and van Leeuwen (1913) on the roll galls of *Fagraea litoralis*, *Loranthus pantandrus*, *Conocephalus suaveolens*, and *Memecylon intermedium*.

A totally different picture of developmental pattern is observed in the pouch galls of *Calycopteris floribundus* developed on the axillary buds. The pouch galls of *Terminalia chebula* (Ananthakrishnan 1978) are restricted to the abaxial surface of the leaves clustered closer to the petiole, a more complicated developmental pattern is observed in the axillary bud galls of *Calycopteris floribundus*. Hence cecidogenesis involves an initial inhibition of growth of the shoot apex by completely destroying it through feeding, resulting in telescoping of the stem, fusion of the young leaf primordia of the axillary bud forming a pouch-like structure leading to the inhibition of normal histogenesis and excessive cellular hyperplasy coupled with cellular hypertrophy of the peripheral gall tissues. An excessive population build-up of *Austrothrips cochinchinensis* and their mode of feeding affect the apical meristem eventually changing the normal growth and organogenesis. This is identical to cecidogenesis in *Acacia* galls induced by *Thilakothrips babuli* resulting in the rosotte gall system, the only difference being the involvement of a number of leaflets showing different grades of development (Raman & Ananthakrishnan 1983b). All gall systems induced by cecidomyiids, cynipids or acarines are in general characterised by inhibition of elongation of the internodes, multiplication of leaves, and an early arrest of apical growth. Jauffret (1978) considers these morphogenetic effects as the consequence of a progressive transformation of meristematic cells into

nutritive cells. *Calycopteris* galls also conform to this category, although the multiplication of leaves is not visible. The intensity of cecidogenetic action in bud galls generally seems to be counteracted by the activity of the normal meristem.

Another notable feature in *Calycopteris* galls is the development of leaf epidermal hairs. Eventhough in normal histogenesis the number of unicellular hairs decreases during maturation, the appearance of dense unicellular hairs on both surfaces of the galls together with stellate hairs on the outer surface even in old galls appears interesting. This addition in number of hairs on the surface of *Calycopteris* galls is a rare phenomenon among thrips galls, which seems to be identical with the development of erineal hairs in acarocecidia; however, it has been confirmed through electron microscopic observations that thrips do not feed on the trichomes in *Calycopteris* galls. Apparently these hairs form favourable breeding sites for oviposition and also for development of the young ones of the cecidozoan.

Tissue Diversity

Cells influenced by insect feeding response cease their normal pattern of differentiation and become cytologically active, organising the nutritive tissues. Meyer (1969) defined the nutritive tissues in insect and mite galls as characterised by abundant cytoplasm, nuclear and nucleolar hypertrophy, rich RNA, and poorly differentiated plastids. Though light microscopic studies did not reveal clearly the presence of nutritive zone (Ananthakrishnan 1978), through electron microscopic studies of three leaf galls on *Casearia*, *Pavetta*, and *Mimusops* induced by thrips, Raman and Ananthakrishnan (1981a) has established a well-defined zone of nutritive cells in thrips galls also. The nutritive cells existing as isolated patches along the lamina are found to extend deeper into the leaf tissues as a result of the sustained feeding action of thrips, a feature also found in homopteran galls. The present studies also have revealed the incidence of definite nutritive tissues in isolated patches, sometimes covering a major part of the tissues lining the gall chamber in the galls of *Calycopteris floribundus*, *Loranthus elasticus*, and *Vitis lanceolaria*. In contrast to this, in *Pothos* galls a well-defined nutritive zone is absent, exhibiting a reduced response to feeding. This is comparable to the primitive galls, in which a well-defined nutritive zone does not exist. The meristematic activity of the gall mesophyll cells near the feeding sites is a visible feature in the galls studied. This is brought about by the transformation of primordial cells into meristematic cells. The number of cell layers constituting the nutritive tissues ranges from 4 to 12, the maximum being found in *Calycopteris* and *Vitis* galls. The characteristic nature of the vacuole morphology in the nutritive tissues with numerous small vacuoles appears similar to the observations of Raman (1981) on thrips galls. The very presence of split vacuoles in the nutritive cells of thrips galls shows a tendency towards organisational patterns similar to meristematic cells. (Coulomb & Buvat 1968, Buvat 1970) suggesting an active state of cell. Another common feature found in the nutritive zone of the galls studied here is the prominent cell necrosis at the feeding site a feature which distinguishes these galls from other homopteran galls, in which cells do not show necrosis. Westphal (1977) has reported the similar incidence of necrosed cells at the feeding spots in the galls induced by mites. Galls serve as centres of nutritional value and the cecidozoans are supposed to derive their nutrition from the host tissues for their development and multiplication. This

kind of a nutritional dependence necessitates the formation of a new 'adaptive tissue' (Maresquelle & Meyer 1969).

Tissue Differentiation

Dedifferentiation of tissues is a common feature in thrips galls though not as conspicuous as in hymenopteran and dipteran galls. Thrips galls generally show an inhibition of normal histogenesis, the primordial mesophyll cells being differentiated to parenchyma cells which exhibit meristematic activity in the nutritive zone. This is evident in the galls of *Calycopteris*, *Loranthus* and *Vitis* while it is absent in *Pothos* galls.

In the present investigation the galls studied do not show any incidence of sclereid differentiation. In *Loranthus* gall tissues the inhibition of sclereid idioblast differentiation which appears in normal leaf tissues is interesting. In normal leaf tissues of *Vitis*, the cells bearing crystals found along the margins of phloem cells is a characteristic anatomical feature found among the genus *Vitis* (Esau 1972). This feature is totally absent in the gall tissues of *Vitis*.

Vascular proliferations as evident in dipteran and hymenopteran galls are found in *Calycopteris*, *Loranthus*, and *Vitis* galls. Differentiation of parenchyma cells of the nutritive tissues to tracheary elements is evident in the galls investigated, connected to the main vascular bundles. Such differentiation of parenchyma cells to vascular tissues is a notable feature met with in abnormal conditions (Sinnot 1960). Similar cases of 'self-perpetuation' were observed in the wounded stems of *Coleus* sp. which involved the redifferentiation of the tissues already differentiated to pith or cortical cells (Lang 1973). In thrips galls, although the vascular elements are displaced (Ananthakrishnan 1978), features such as the elongation of tracheary element showing annular to scalariform thickenings are restricted to such complex pouch galls of *Schefflera* (Krishnamurthy et al. 1975), and *Calycopteris*. Thus the tissue differentiation in galls is predominantly in relation to the position and nature of the gall-maker.

Nuclear Hypertrophy, Cellular Hyperplasy and Hypertrophy

Nuclear gigantism is not uncommon among gall tissues. In the present observation nuclear hypertrophy calculated in terms of volume reveals an abnormal magnification in all the four gall tissues examined. Nuclear hypertrophy is maximum in the nutritive cells, the least being observed in gall peripheral cells, which show linear and elongated nuclei. An overall increase in volume of the nuclei in gall tissues compared to the nuclei in normal tissues appears interesting (tables 2–5). A peculiar pattern of nuclear hypertrophy exhibiting an increase in volume and size in each cell layer starting from the nutritive zone and reaching its peak in the last layer of the nutritive tissue and thereafter showing a gradual decrease in cells farther away from the nutritive zone in *Loranthus* galls, irrespective of the age of the gall is a notable feature (figure 2). Similar observations were made by Hesse (1968) on the galls of *Andricus marginalis* and *Neuroterus quercusbaccarum* which also exhibited a maximum ploidy in nutritive cells with a decrease towards the peripheral gall tissues. Mayer (1950), while studying the ratios of gall and normal nuclear sizes in nine different galls, found a considerable increase in size of the gall nuclei and related it to the increase in RNA and proteogenesis. Recently, electron microscopic observations of the galled nuclei in thrips galls showed enlarged and lobed outline for the galled nuclei together with hypertrophied nucleoli (Raman 1981) is also

compatible to the present investigation on nuclear hypertrophy.

Hyperplasy can be considered as the most important histogenetic process in cecidogenesis. This brings about diverse conditions like abnormal direction of cell division, unequal cell division, and division of nucleus without simultaneous division of cells thus resulting in multinucleate giant cells as in nematode-induced galls. The present investigation has revealed maximum hyperplasy in *Calycopteris* galls constituting 60–70 layers of cells followed by *Vitis* and *Loranthus* galls. Anticlinal divisions of the peripheral gall tissues away from the gall chamber are another feature found in fold galls, hypophyllous and epiphillous roll galls constituting the rolling or folding of the leaves. Because of the excessive division of the mesophyll cells, the midrib region of the galls of *Vitis* loses its identity.

The proliferation of upper epidermis in epiphyllous galls and of lower epidermis in hypophyllous galls is evident in *Loranthus* and *Vitis* galls, more prominently in the latter. Arnold (1969) similarly reports that many mite galls arise as a result of cell divisions induced by the epidermis. Epidermal cells normally considered to be unsuitable for divisions (Sinnot 1960) are found to divide periclinally adding to the thickness of the gall in *Vitis*, whereas in *Loranthus* this is evident in isolated feeding sites. Hypodermal proliferations as evident in *Piper* and *Schefflera* galls (Raman 1981) are absent in *Vitis* galls as the hypodermis is found to be almost intact as in normal conditions but exhibiting hypertrophy.

Insect galls show a controlled cell division influenced by the cecidozoan and can be considered to acquire autonomy from their host organs (Rohfritsch & Shorthouse 1982) in contrast to the uncontrolled divisions found in crown galls. In thrips galls, most cellular proliferations occur in the nutritive cells or in their vicinity which forms the centre of cecidogenetic influence. Similar observations of restricted activity of cellular proliferations in the nutritive cells adjoining the larval chamber and of almost non-existence near the point of attachment to the normal tissues were made by Houard (1903) and Garrigues (1954).

Cellular hypertrophy, a principal cecidogenetic phenomenon, exists predominantly in thrips galls. In the present investigation, an overall statistical analysis of the degree of hypertrophy in the gall tissues in comparison with normal cellular dimension has shown a significant rate of cellular hypertrophy in all the gall tissues such as the nutritive cells, epidermis, vascular tissues, and peripheral parenchyma cells. In general the cells away from the nutritive zone exhibit maximum cellular hypertrophy in all the galls studied. This is identical to the observations of Maresquelle and Meyer (1965), who showed that the rate of hypertrophy appeared to be greater in mesophyll regions away from the nutritive zone during the developing stages of many leaf galls. Hypertrophied nature of the phloem parenchyma cells in *Vitis* galls are compatible to the observations made by Karny and van Leeuwen (1913) and Krishnamurthy et al. (1975) in the thysanopteran galls of *Loranthus* and *Schefflera* respectively. Such a cellular rearrangement in diverse grades in the gall tissues ultimately brings about the organisation of a "form" deviating from the original morphology of the organ involved. Hyperplasy and hypertrophy are among the principal adaptive phenomena met with in thrips galls (Ananthakrishnan 1983).

(b) Histochemistry of the Gall Tissues in Comparison with the Normal Leaf Tissues

Observations on the activity of the enzyme acid phosphatase in the galls reveal that

they are mostly confined to the nutritive tissues in all the stages of development in the galls of *Calycopteris*, *Loranthus*, and *Vitis* though interestingly in *Pothos* gall systems, the activity of this enzyme is seen only in the younger stages. This kind of a predominant activity of acid phosphatase in the nutritive tissues has been observed in acarine, dipteran, and cynipid galls (Westphal 1968a, Bronner & Meyer 1972, Christiane 1966, Bronner 1973, 1974). Yet another feature is the wall-bound activity of this enzyme in the cells of the nutritive zone. The localisation of the acid phosphatase activity along the cell wall and plasmalemma of the nutritive cells of thrips galls appears identical to the observations of Bronner (1973, 1974), who suggested the possible function of these enzymes in facilitating intercellular transport of carbohydrates towards the nutritive zone. The stability in the activity of acid phosphatase in the nutritive layer and associated vascular tissues, similar to conditions in the cecidomyiid and cynipid galls (Christiane 1966), indicates a higher metabolic activity in cells very close to the locus of insect incidence.

The gall tissues, especially those of the nutritive zone, and the associated vascular tissues in general show a hyperactivity of proteins. This feature is of a higher intensity compared to the mesophyll and vascular tissues of normal leaves. The incidence of protein in the nutritive tissues has been reported by Westphal (1972a) in acarine galls, and Rapp and Krist (1974) in the *Mikiola* galls on *Fagus*, and they have suggested that the accumulation of protein in the nutritive cells helps the cecidozoa in their growth and development. Wounding, known to accelerate protein synthesis (Kahl 1974), appears relevant in the context of thrips galls since wounding as a result of feeding damage is an important prerequisite in the development of thysanopterocecidia (Ananthakrishnan 1981). This in conjunction with the higher rate of incidence of polysomes and protein fibres (Raman & Ananthakrishnan 1983a), reflects the importance of protein accumulation and localisation in nutritive cells.

Lipid droplets which occur localised in the nutritive tissues of *Loranthus* and *Vitis* cells, especially in the later stages of development, are wanting in the healthy tissues as well as in the gall tissues of *Vitis* and *Calycopteris*. In *Pothos* and *Loranthus* galls, they occur in the cells away from the nutritive zone also. This abundance of lipids in the nutritive tissues is a feature shared with many different galls produced by cynipids (Bronner 1975, 1980) and cecidomyiids (Jauffret & Le Ret 1975). The concentration of lipids in nutritive layers and the gradual decline in the quantity of lipids towards the cells away from the nutritive zone are comparable to galls induced by cynipids. As stated by Kahl (1982), the presence of lipids in the nutritive tissue could be related to the wounding, which triggers a dramatic increase in lipid biosynthesis. The present investigation on the lipid droplets in the nutritive tissues well substantiates the statement of Ananthakrishnan (1983) on the increase in lipids and carbohydrates in galled tissues enabling the thrips to maintain their life activities for a considerable period.

Accumulation of starch in the nutritive tissues occurs during the later stages of ontogeny of the gall in the leaf gall systems (*Loranthus*, *Pothos* and *Vitis*), while starch appears localised in the cells away from the nutritive zone in the axillary bud gall systems (*Calycopteris*). All these show a characteristic pattern of disposition of starch in an increasing gradient towards the cells away from the nutritive zone, as also known in cynipid galls (Bronner 1971b, 1975), and nematode galls (Orion & Bronner 1973). The appearance of starch grains especially during the later stages of cecidogenesis

appears significant. As suggested by Bronner (1971a) a possible diffusion of the soluble saccharides produced by starch hydrolysis towards the nutritive tissues where they are broken down to monosaccharides or to smaller molecules may exist in thrips galls also.

A hyperactivity of peroxidase and polyphenoloxidase is evident throughout the gall tissues in all stages of development when compared to the healthy tissues. Vigorous activity of these enzymes is seen in the nutritive zone cells and cells farther away, which suggests a triggering of the synthesis of these enzymes by the feeding stimulus of the cecidozoan and subsequent wound initiation. Increased PO activity has been demonstrated cytochemically in galled tissues of several plants infested with *Meloidogyne incognita*, *Acrita* (Myuge 1956). A high incidence of peroxidase and polyphenol oxidase was observed in *Phylloxera* galls by Schaefer (1972, 1980), and in various other galls by Tandon and Arya (1982), Tayal et al. (1981), and Purohit et al. (1979a). The cells adjacent to a wound cope with the parasite attack by the luxurious formation of various kinds of phenols (Kahl 1982). The oxidation of phenolics to quinones is accomplished by PPO and peroxidases (Levin 1971). This activation of polyphenol oxidases and peroxidases in infected tissues is just as widespread as parasite-stimulated phenolic synthesis. Salivary secretions of thrips have been shown by Kloft and Ehrhardt (1959) to penetrate plant tissues when the insects feed, but little or nothing is known of their composition. The PPO and PO are also involved in reaction leading to the darkening of the plant tissues after injury (Uritani & Oba 1978), which are comparable to the numerous dark areas found near the feeding spots in thrips galls.

The present studies pertaining to the histochemical localisation of condensed tannin precursors in thrips gall tissues appear to be a new feature, reported for the first time in gall tissues. Among the four galls studied the gall tissues of *Calycopteris* and *Vitis* exhibit their presence in the nutritive tissues, epidermal cells, vascular tissues as well as, in the gall peripheral tissues which are totally absent in gall as well as in healthy tissues of *Loranthus* and *Pothos*. Another interesting feature is their localisation in the epidermal hairs of both normal leaves and galls. The presence of condensed tannin precursors, the major polyphenols in the leaves of mature plants, has been reported by Bell (1968) and the possible function of these polyphenols as chemical barriers to penetration and colonization of fungal pathogens has been discussed by Mace and Howell (1974).

The presence of condensed tannin precursors in *Calycopteris*, and *Vitis* galls as well as in healthy tissues for which the general tannin histochemical tests also exhibit positive reactions in the same regions where the condensed tannin precursors are seen, shows that the type of tannin present in them is the condensed tannin which is non-hydrolysable. In contrast to this the *Loranthus* tissues do not show the presence of condensed tannin precursors, suggesting that they contain hydrolysable tannins. The localisation of condensed tannin precursors in the epidermal hairs of the galls as well as those of the normal leaves appears compatible with the observations of Beckman et al. (1972). They concluded that the phenolic type compounds observed in the trichomes of many species appear in the terminal portions and in response to certain stimuli like light, and that the material is dissipated quickly by moving into the epidermal cells. This type of response to the light is not evident in the trichomes of *Calycopteris*. Beckman et al. (1972) established that the phenols are not only involved in defence mechanisms, but also in wound healing and in the general synthesis and regulation of the important

growth hormone, indole acetic acid, a feature which could be related to the excessive cellular hyperplasy in *Calycopteris*.

The presence of tanniniferous cells in many thrips galls is reported by Ananthakrishnan (1978). Hyperactivity of tannin is evident in the gall tissues compared to the normal tissues, but absent in the healthy and gall tissues of *Pothos scandens*. A notable feature is the presence of tannin in the healthy tissues right from the young age, but not as concentrated as in gall tissues. Being localised in the nutritive tissues as well as in the promordial mesophyll cells of young leaves the site at which thrips feed, it may be presumed that thrips utilise tannin for feeding. Many phenols reside in plant tissues in a simple form (Chlorogenic acid, Caffeic acid) or as complex ones, as tannins (Levin 1971). Even though the phenolics are commonly regarded as feeding deterrents for the phytophagous insects (Feeny 1976, Levin 1971), recent observations of Bernays et al. (1980), Bernays and Chamberlain (1980), Bernays (1982), and Bernays and Woodhead (1982) have revealed the plant phenols as being utilized by acridids. The major part of the phenols consumed is retained by the insect, bound to the cuticle. This is comparable to the present investigation where consumption of phenolics in the form of tannins by thrips from the nutritive tissues of the galls and the young healthy tissues during cecidogenesis is possible. Such availability and utilisation of tannin by the gall-maker from its host plant could be considered an adaptive strategy for the cecidozoan.

In order to achieve a functionally efficient organic compromise by differential elaboration of the tissues in gall systems, the functional elaboration in the cells closer to the feeding sites of the gall-maker (as supported by the higher enzyme activity, incidence of proteins, starch, lipids, coupled with cytological specialisation), and the elaboration in terms of morphological criteria of the cells away from the nutritive zone (the cells away from the nutritive zone being nearly twice the size of the cells of the nutritive zone) appear impressive.

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Proc. Indian natn. Sci. Acad. **B51** No. 5 pp 457–460 (1985)

Synthesis of Haemolymph Proteins in the Desert Locust, *Schistocerca gregaria* Forsk.

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The kinetics of the synthesis of haemolymph proteins (HP) in the desert locust was studied by using labelled leucine. The synthesis of HP showed a lag time of release of HP in the haemolymph, of 32.8 min in male and 39.6 min in female locusts. The rate of HP synthesis ($t_{1/2}$) was 74.3 ± 17.1 min in male and 28.1 ± 9.4 min in female locusts. The higher rate of HP synthesis in female locusts was also observed by using dual labelling technique.

Key Words: Desert locust, *Schistocerca gregaria* Forsk., Synthesis, Proteins

Introduction

A survey of recent reviews (Wyatt & Pan 1978, Chen 1978, Engelmann 1979, Hagedorn & Kunkel 1979) show that there is an acute dearth of information on the kinetics of haemolymph protein (HP) synthesis in insects. Limited work on the kinetics of protein synthesis which has been reported so far, do not provide information on the basic mechanism of their synthesis (Dinamarca & Levenbook 1966, Levenbook & Krishna 1971, Williams & Birt 1972). The present study therefore attempts to analyze kinetics of incorporation of labelled leucine into HP of the adult desert locust, *Schistocerca gregaria* Forsk.

Materials and Methods

Experimental Animals

Insects used in this study were seven day old adult locusts belonging to the gregarious phase. These were obtained from laboratory colony maintained according to Mehrotra and Rao (1966).

Chemicals

^{14}C(U) L-leucine (290 mCi/mMol) and ^{3}H(G) L-leucine (3300 mCi/mMol) were procured from Isotope Division, The Bhabha Atomic Research Centre, Trombay.

All other chemicals used were of analytical grade and were procured locally.

Experimental Procedure

Synthesis of HP was studied by following the incorporation of ^{14}C-labelled leucine in the protein fraction. Each locust was injected with 1.0 μCi of ^{14}C-leucine and/or 10 μCi of ^{3}H-leucine contained in 1 μMol of leucine. The amount of leucine thus injected were about 5 to 7 times higher than its natural

concentration in the haemolymph (Kulkarni & Mehrotra 1970). The objective of injection of high concentration of leucine was to study the synthesis of HP at a concentration of leucine which was not rate limiting.

In experiments involving dual pulse labelling, ^{14}C-leucine was injected initially; three hr later, ^{3}H-leucine pulse was given to study incorporation of both isotopic forms of leucine up to 6 hr after ^{3}H-leucine injection.

The incorporation of labelled leucine was determined in trichloroacetic acid (TCA) precipitable fraction of the haemolymph 1, 2, 3 and 6 hr after injection of the label. The TCA precipitable fraction was taken to represent the protein in the haemolymph.

Processing of Haemolymph Samples

Samples (5 μl) of haemolymph collected at definite time intervals were processed for assay of total and TCA-precipitable activity as described earlier (Gujar & Mehrotra 1982).

Assay of Radioactivity

The radioactivity in the samples was assayed with the help of Packard Tricarb Liquid Scintillation Spectrometer (Model 3255) using 5 ml of toluene-based scintillation mixture prepared according to Mans and Novelli (1961).

The data on radioactivity was expressed in disintegrations per min (dpm) after correcting for background and quenching as outlined by Baillie (1960). In case where double labelling technique was used, the resolution of the radioactivities of the two isotopes was done by using simultaneous equations (Snipes & Iengemann 1971).

Estimation of Proteins

Proteins present in the haemolymph samples were estimated by the method of Lowry et al. (1951) at 610 nm by using serum albumin fraction V (Sigma) as a standard protein.

Results and Discussion

The data on incorporation of labelled leucine into HP (TCA-precipitable dpm/5μl haemolymph) were used to calculate various kinetic constants according to Haschemeyer (1973). The data on the incorporation of labelled leucine into HP when subjected to kinetic analysis according to Haschemeyer (1973) gave a straight line relationship. The kinetic constants were calculated from the data by a least square method using a computer programme (Hindustan Computer Micro 2200). (Mehrotra, Unpublished results). From the data presented in table 1, it would be seen that the lag time for release of HP from the site of its synthesis in the haemolymph was found to be 32.8 min in male and 39.6 min in female locusts. These results on lag time of HP secretions are similar to those reported in this species by Nakat (1974). Similar results on lag time of HP secretion of about 10–50 min have also been reported in a few insect species earlier (Faulkner & Bheemeswar 1960, Skinner 1963, Martin et al. 1969).

The half time ($t_{1/2}$) obtained for synthesis of HP shows that there were insignificant differences between two isotopes used (table 1). However, significant differences between two sexes were seen, statistically, $t_{1/2}$ for synthesis being 74.28 ± 17.07 min in male and 28.11 ± 9.43 min in female locusts. These results therefore suggest that rate of protein synthesis in female locusts is higher than that in male locusts. In fact, the rate of protein synthesis expressed in *ksyn* values is found to be 0.0096 min^{-1} in male and 0.0269 min^{-1} in female locusts (table 1).

In order to investigate further into the differences of *ksyn* values between sexes, dual pulse labelling technique was followed. In the present investigations, involved pulse labelling with two isotopic forms of leucine viz. ^{3}H and ^{14}C at 3 hr interval. The analysis of data of dual pulse labelling experiment shows that *ksyn* values were 0.016 min^{-1}

Table 1 *Kinetic constants for* in vivo *synthesis and secretion of haemolymph proteins in desert locust,* Schistocerca gregaria *Forskal*

Isotope incorporation	Male			Female		
	*lag time (min)	$t_{1/2}$ synthesis (min)	A=6 At=3	*lag time (min)	$t_{1/2}$ synthesis (min)	A=6 At=3
^{3}H leucine	12.0	58.51 (0.0118)	1.173	24.0	29.56 (0.0234)	1.031
^{14}C leucine	50.4	71.98 (0.0096)	1.403	42.0	18.04 (0.0384)	1.008
^{3}H leucine**	36.0	92.34 (0.0075)	1.181	52.9	36.73 (0.0188)	1.023
^{14}C leucine**	—	87.02 (0.0079)	1.131	—	83.14 (0.0083)	1.037

*lag time determined by linear extrapolation to zero activity from the period of most rapid accumulation
**based on experiments involving ^{14}C and ^{3}H-leucine together
Figures in parenthesis are *ksyn* (min^{-1}) values

in male and 0.02 min^{-1} in female locusts. Although *ksyn* value in female locust was higher than that in male locust, the difference between these values appear to be less evident in comparison to that observed in single pulse labelling studies. The data on *ksyn* in dual pulse labelling however appear to be close to true value because of elimination of error contributed by lag time (table 1).

The probable differences in protein synthesis rates in two sexes are also seen in the half life values of leucine disappearance in the haemolymph of locusts. The results of analysis of data provided by the single and dual pulse labelling experiments are presented in table 2. From the data in table 2, it would be seen that $t_{1/2}$ values for disappearance of leucine from haemolymph were 118.3 ± 17.6 min in male and 106 ± 22.6 min in female locusts. These values are intermediates between the fast and slow phases of leucine disappearance observed by Nakat and Mehrotra (1981). The higher rate of loss of leucine in female locusts thus explains the higher rate of protein synthesis.

The similar higher value of synthesis of HP in female locust in comparison to male has also been reported in *S. gregaria* (Nakat 1974). The higher rate of protein synthesis in female might be due to the greater requirement of HP for growing reproductive organs in female locusts (Injeyan & Tobe 1981).

Table 2 *Half-life values (min) of loss of labelled leucine in desert locust,* Schistocerca gregaria *Forskal*

Isotope incorporation	Male	Female
^{3}H leucine	129	112
^{14}C leucine	128	125
^{3}H leucine*	98	81
^{14}C leucine*	489	848

*Based on experiment involving dual pulse labelling studies

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Proc. Indian natn. Sci. Acad. B51 No. 5 pp 461–467 (1985)

Ontogenetic Variations in Carbohydrate, Lipid and Protein Contents in *Trabala vishnou* Lef. (Lepidoptera: Insecta)

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Day-to-day quantitative changes in carbohydrates, lipids and proteins contents during embryonic and post-embryonic development of *Trabala vishnou*, as also, the age and sex-related changes in these biochemical constituents have been studied. The female larvae, pupae and adults showed higher contents of carbohydrates and proteins as compared to their male counterparts. However, lipid contents were more in the various developmental stages of the male than of the females. The concentrations of all the biochemical constituents examined were more in the pre-moult than in the post-moult period. The carbohydrates, lipids and proteins, accumulated during the larval stages, were utilized during metamorphosis. The relationship between the quantitative changes of carbohydrates, lipids and proteins and various physiological and morphological events associated with development have been discussed.

Key Words : *Trabala vishnou* **Insecta Ontogenic variations, Carbohydrates, Lipids, Proteins**

Introduction

Studies on carbohydrates, lipids and proteins during insect development could indicate the level of activity and energy requirements during a given phase of development. The role of various biochemical constituents during insect development has been reviewed by Chen (1971). Most of the earlier studies pertain to a few arbitrarily chosen stages, like egg, mature larva, pupa and adult and further the studies on the sex-specific differences in these biochemical constituents have been restricted mainly to the adult stage.

In the present investigation, day-to-day changes in reducing carbohydrates, lipids and soluble proteins have been studied during the whole process of ontogeny of *Trabala vishnou*. In addition, the sex-specific differences in these biochemical constituents at larval, pupal and adult stages have also been studied. We have tried to correlate the chemical body composition to such phenomena as embryogenesis, acdysis, spinning, adult emergence, egg-laying and mating, etc.

Materials and Methods

The culture of *T. vishnou* larvae was maintained in an incubator on the leaves of castor, *Ricinus communis* at 28±2°C and 70% RH.

All the developmental stages of known chronological age were taken for analysis and were dried at 80°C to constant weight (48 hr), kept in a desiccator for 24 hr and weighed. No morphological distinctions were present to differentiate sex in the larval stages. However, in the final phase of Vth instar, larvae were sexed on the basis of their live weight (Gakhar 1983).

Total reducing sugars were estimated from ethanolic extract with the help of Dreywood's anthrone reagent using glucose as a standard (Morris 1948). For the quantitative determination of lipids, known dry weights of various stages were extracted with chloroform : methanol (2 : 1 v/v) for 24 hr (Folch et al. 1957). The proteins were determined by the method of Lowry et al. (1951).

All the data were subjected to students' 't' test to determine statistical significance.

Results and Discussion

Changes in the concentration of carbohydrates, lipids and proteins during the various stages of development are shown in figures 1–4.

Reducing Carbohydrates

The concentration of reducing sugars declined up to Day-3 by 33.8% ($P<0.01$) of embryonic development. It more than doubled on Day-4. Thereafter, it again declined continuously by about 75% ($P<0.01$). The breakdown of yolk reserve, which actively occurs during early embryogenesis requires utilization of carbohydrates, their depletion occurs initially (Chen 1971). The breakdown of yolk, however, results in the release of several products which, in turn, increase the carbohydrates on Day-4. The subsequent decline of carbohydrates may be related to the requirement of glucosamine in the formation of chitin for the pharate larva besides normal metabolic requirement (Chefurka 1965).

During the course of every larval instar, the sugar contents were more in the final phase of preceding instar as compared to the initial phase of the succeeding instar ($P<0.01$). The possible reason may be that carbohydrates are used for chitin synthesis or converted into reserve carbohydrates like trehalose or glycogen. Chang et al. (1964) observed a reverse correlation between the reducing carbohydrates and glycogen. Moreover, Tate and Wimer (1974) advocated that the soluble carbohydrates which accumulate in the larval stages, are utilized during metamorphosis.

Domroese and Gilbert (1964) on *Hyalophora cecropia* and Campbell et al. (1976) on *Sitophilus* suggested that the respiratory quotient (RQ) was more in the larval stages than in the pupal and the adult stage. It was, therefore, suggested that the larval stages use reducing carbohydrates as respiratory substrates, whereas, the pupal and the adult stages utilize non-carbohydrate sources (mainly lipids).

During metamorphosis, carbohydrate contents increased slightly during first four days, thereafter, it generally had a declining trend. This is in agreement with the findings of Singh and Sinha (1977). Tate and Wimer (1974) have shown that the reducing carbohydrates are utilized more for the synthesis of proteins and other carbohydrate moieties than for the process of energy production during pharate adult development.

During the adult life, carbohydrates concentration declined by about 45% in the male moth and by 70% in the female moth ($P<0.01$) up to the day before death. This may be due to active flight by the male in search of female and due to egg laying in the female. Domroese and Gilbert (1964) suggested that the adult metabolize both reducing carbohydrates and lipids.

The higher level of reducing sugars and soluble proteins in the female larvae, prepupae, pupae and adults than in the males ($P<0.01$) may be due to its necessity for

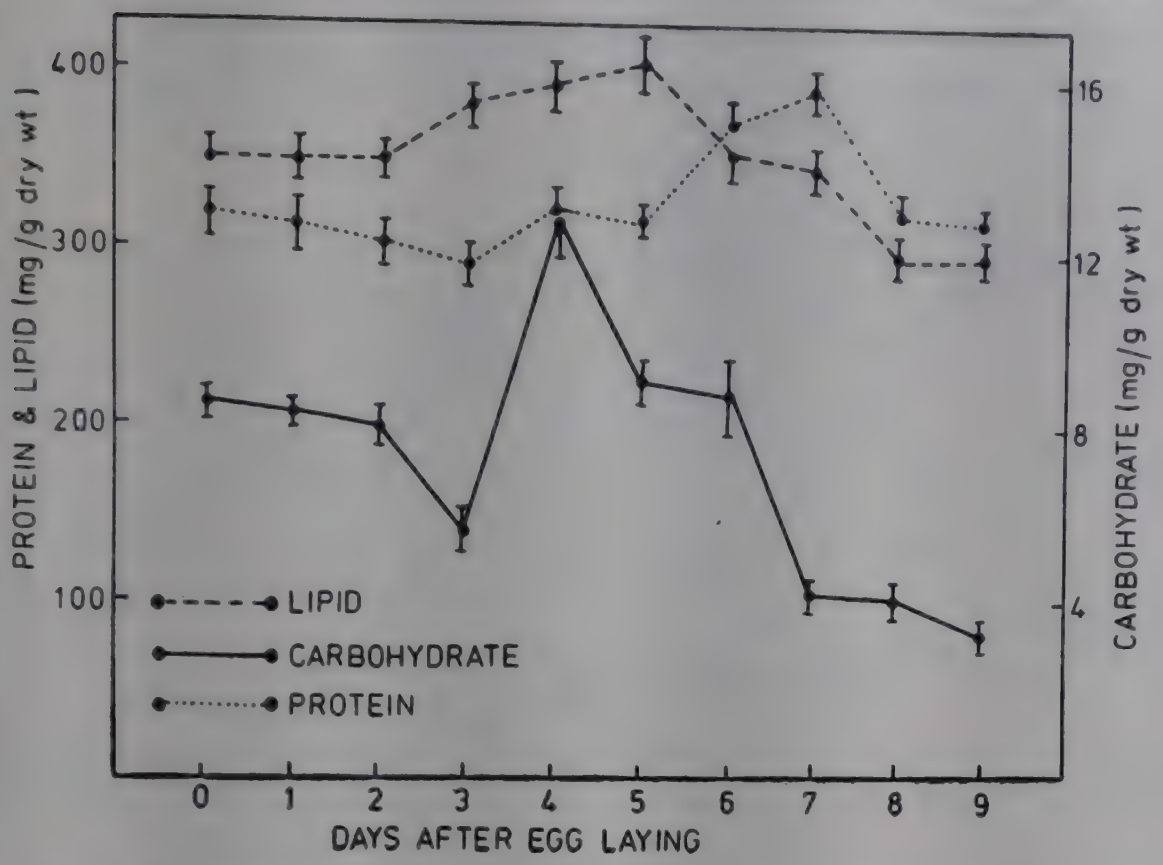

Figure 1 Changes in carbohydrates, proteins and lipids during embryogenesis of *T. vishnou*

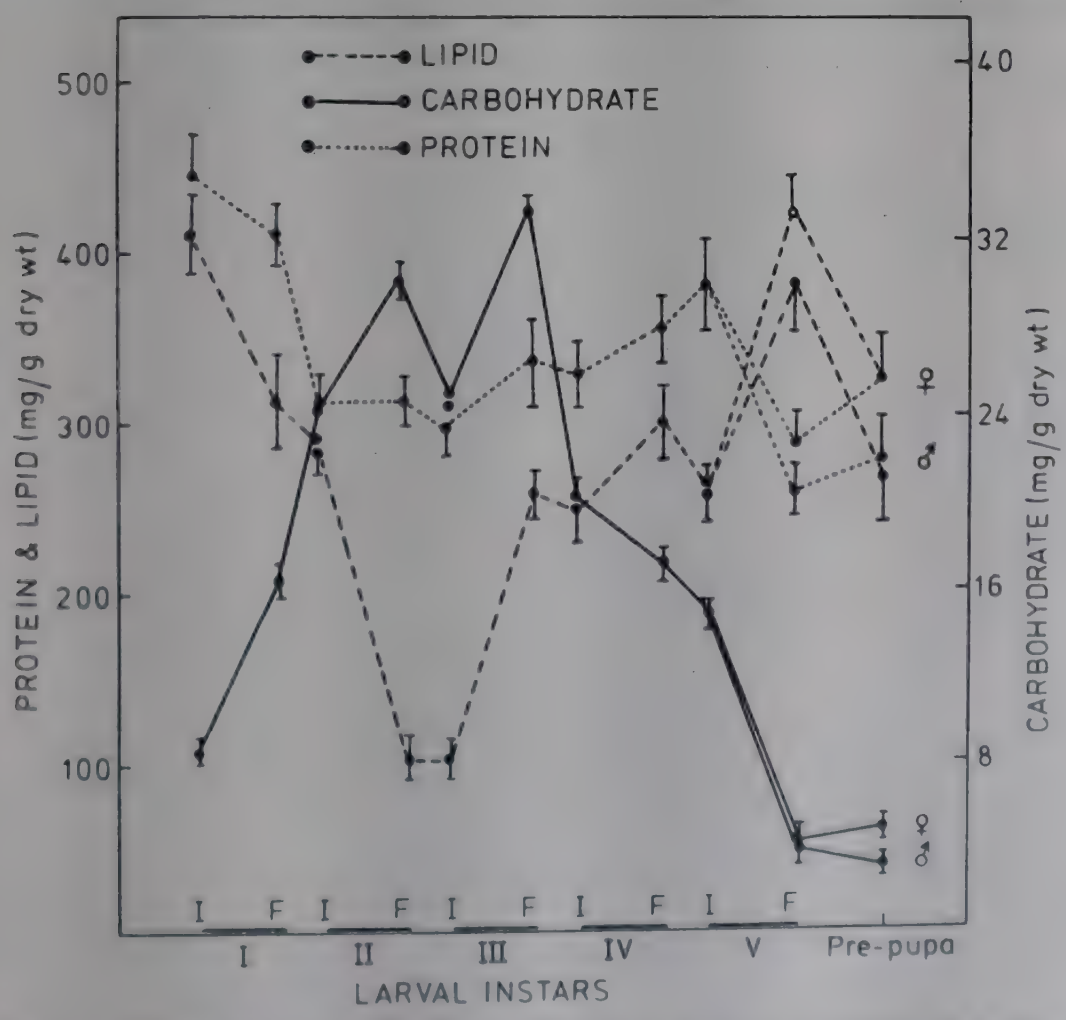

Figure 2 Changes in carbohydrates, proteins and lipids in various larval stages of *T. vishnou*

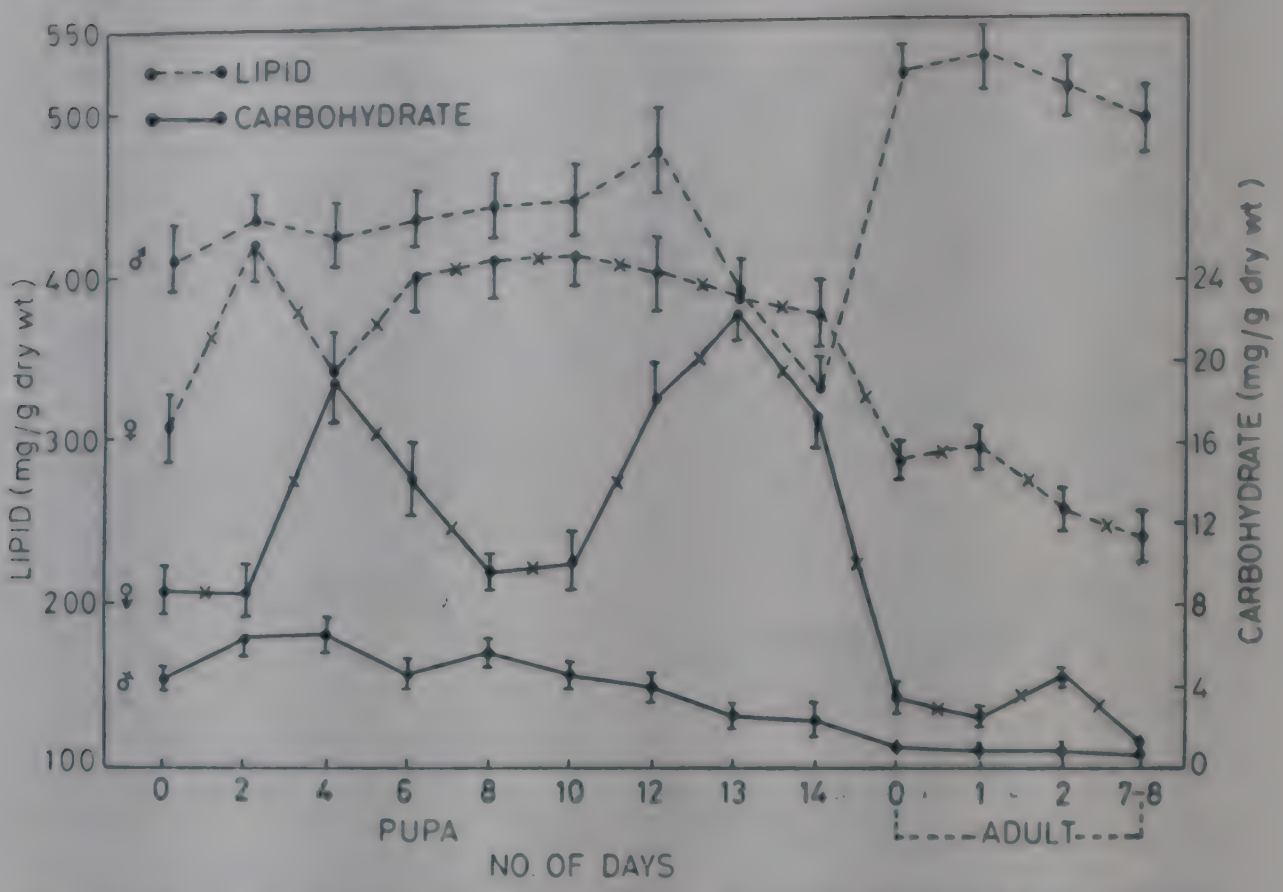

Figure 3 Changes in carbohydrates and lipids during metamorphosis and adult life of *T. vishnou*

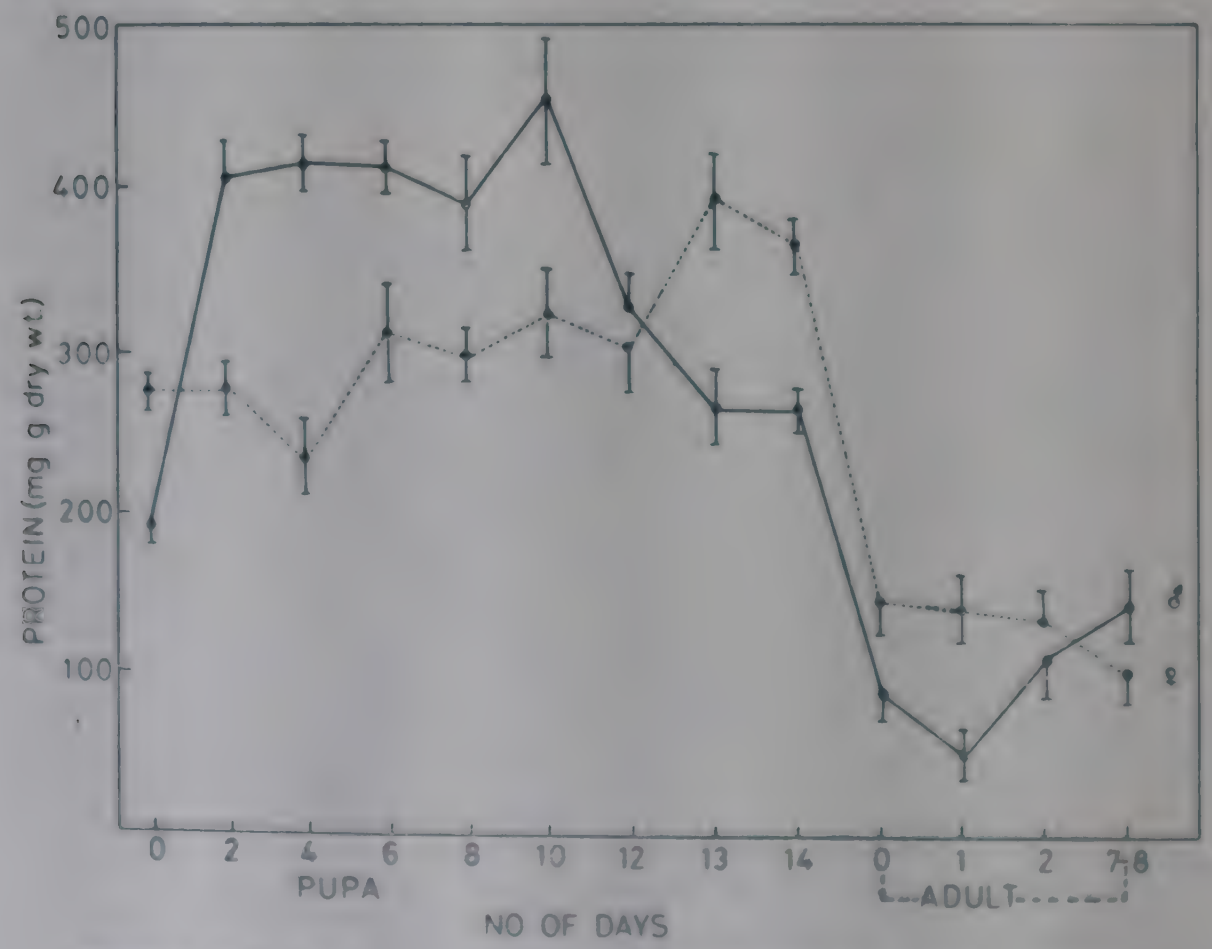

Figure 4 Changes in proteins during metamorphosis and adult life of *T. vishnou*

higher rate of metabolism owing to the maturation and development of oocyte in the female.

Lipids

The concentration of lipids increased by 15.7% ($P<0.05$) during first five days of embryogenesis. However, it declined by 25% ($P<0.01$) during the following days of embryonic development because lipids provide the primary source of metabolic energy during embryogenesis (Gilbert 1967). These findings are in agreement with those of many workers (Babcock & Rutschky 1961, Fast 1964, Gilbert 1967).

During larval growth, the lipids decreased by 75% up to the initial phase of Instar III. Thereafter, it increased by four-fold during subsequent larval instar ($P<0.01$). This is in conformity to the earlier reports of D'Costa and Birt (1966), Janda et al. (1966), Mukerji and Guppy (1973) and Singh and Sinha (1977). Such a high concentration and increase in the lipid contents during the successive larval instars could be due to its formation through inter-conversions from free amino acids, proteins and carbohydrates which show decline during this period (Wyatt 1967, McCabe & Bursell 1975, Gakhar 1983, Gakhar & Maleyvar 1984a,b). Increased concentration of lipids before moulting appears to be related with the increased rate of oxygen consumption during this phase (Gilbert & Schneiderman 1961). Declining level of lipids in the post-moult period may be associated with their utilization in the complex biochemical process related to moulting.

The results of the present study indicate a drop in lipids during pre-pupal development ($P<0.01$). This is similar in other Lepidopterans, *Hyalophora cecropia* (Bade & Wyatt 1962) and *Lambdina fascellaria* (Dunphy et al. 1977). This phenomenon is, in general, a characteristic of holometabolous insects where the energy for pupal transformation is derived from lipids, especially reserves of triglycerides accumulated during the larval stages (Fast 1964).

The quantity of lipids increased during the early pupa ($P<0.05$) which supports the findings of Pant et al. (1974) who reported that the biosynthesis of lipids takes place in the pupa but at a slower pace. Thereafter, it declined sharply in both the sexes ($P<0.01$). This may be owing to its utilization as respiratory substrate.

During the adult life, there was a continued decline in the lipid contents with ageing in both the sexes. These results are in agreement with the findings of Nowosielski and Patton (1965) in *Acheta*; Sharma and Sharma (1979) and Sidhu et al. (1980) who also reported decline in lipid contents with advancing age of Bruchids.

The reason attributed for higher concentration of lipids in the males ($P<0.01$) is said to be correlated with their mating behaviour. As the male moth has to fly great distances in search of the females, it needs more reserves (Gilbert 1967).

Proteins

The level of soluble proteins declined by about 6% ($P < 0.05$) during the first three days of embryogenesis. Thereafter, it generally increased up to Day-7 and then declined by 19.9% ($P<0.01$) on the last two days of embryonic development (figure 1). The best explanation for the decrease in the concentration of protein with the onset of embryogenesis is the rapid increase in free amino acids pool during early days of embryogenesis (Maleyvar & Gakhar 1982). There must be breakdown of yolk proteins to release free amino acids, then this is followed by the active phase of histogenesis and differentiation. So for the protein synthesis, this would use the amino acids pool and cause a rise in the amount of soluble proteins. This must be the reason for such rise in soluble proteins during Days 5, 6 and 7 of embryogenesis. It could well be coincidental that most of

the free amino acids pool appears to be mopped up during this period (Maleyvar & Gakhar 1982). A similar trend of protein use and resynthesis has been noted in *Locust* and *Sphaerodema molestum* (Indira 1963, McGregor & Loughton 1974).

The level of proteins was generally high prior to moulting in all, but the last instar ($P<0.01$). This may be due to the appearance of new proteins associated with the moulting process only (Chen 1971, Firling 1977). Declining level of all the biochemical constituents in the post-moult period could also be partly attributed to their loss in the body fluids during moulting, exuviae and faecal waste (Gakhar 1983, Gakhar & Maleyvar 1984c).

The concentration of soluble proteins broadly increased during metamorphosis. Protein synthesis has also been reported in several species with different food habits, such as *Lucila* pharate adults of *P. regina* (Dinamarca & Levenbook 1966). The soluble protein build up during pupal stages of *T. vishnou* may be attributed to the metabolism of glycogen or chitin as suggested by Tate and Wimer (1974) in case of *P. regina*.

During adult life the concentration of soluble proteins declined within 24 hr of emergence in both the sexes. Thereafter, the increase in the protein contents in male moth may be due to the process of degradation during senescence (Gakhar & Maleyvar 1984d). However, the decline in the protein contents in the female moth on Day-8 may be attributed to the process of egg laying. The female larvae, pre-pupae and adults had more quantity of soluble proteins than their male counterparts ($P<0.01$). However, no conclusion could be drawn in this regard during metamorphosis.

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Proc. Indian natn. Sci. Acad. B51 No. 5 pp 468–482 (1985)

Wind and Insect Pollination in a Monoecious and a Dioecious Species of Euphorbiaceae

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(Received 15 April 1983; after revision 20 July 1984)

Wind as well as insects mediate pollen transfer in the monoecious species, *Croton bonplandianum*, and in the dioecious species, *Phyllanthus pinnatus*, but wind is superior. *P. pinnatus* being obligately outcrossing, requires its pollen to be carried to relatively longer distances, while for the pollen of *C. bonplandianum* such a need is not so stringent as the plants are adapted for reproduction both by geitonogamy and xenogamy. The pollen characters and the daily pollen release timings of the respective taxa are accordingly designed. In *C. bonplandianum*, the pollen grains (48 μm; fall velocity 8.7 cm/sec) are released at times (1600–1700 hr) when the weather conditions are unfavourable for their effective dispersal by wind, while in *P. pinnatus*, they (25 μm; fall velocity 1.44 cm/sec) are shed at times of pollen (0700–1000 hr) when turbulent conditions develop promoting effective wind transport. Thus, the selective pressures favouring the timing of pollen release in dioecious and monoecious anemophiles are not one and the same.

Insect pollination in *P. pinnatus* is accomplished by ants (*Camponotus*) and flies (*Sarcophaga, Musca, Rhyncomya*), and in *C. bonplandianum* by thrips and flies (*Musca, Chrysomya*).

Key Words: Pollination, Wind, Insects, Pollen release, *Croton, Phyllanthus*, **Euphorbiaceae**

Introduction

A hand-in-hand development of wind pollination and unisexuality is generally acknowledged (Grant 1951, Leppik 1964, Baker & Hurd 1968, Kaplan & Mulcahy 1971). There are reports of the visits of pollen-consuming and pollen-getting insects to the flowers of such plants more or less adapted for anemophily (Kerner 1904, Knuth 1906-9, Porsch 1956, Goot & Grabandt 1970, Sharma 1970, Pojar 1973). Meeuse (1978) asserted that mere registration of insects to apparently anemophilous plants has no bearing on the principal issue whether entomophily is effective unless 'cross visits' between male and female blossoms are established. Studies in this direction demonstrating the consistent contribution of insects to pollination, in reputedly anemophilous species have appeared recently (Stelleman & Meeuse 1976, Stelleman 1978, Meeuse 1978).

We report here the situation in *Croton bonplandianum* Baill., a monoecious taxon,

and in *Phyllanthus pinnatus* (Wight) Webster, a dioecious taxon of Euphorbiaceae, in which pollination is accomplished by wind as well as insects.

Materials and Methods

Several natural populations of these plants occurring in and around Visakhapatnam (17° 42'N and 32° 18'E) were chosen for the present study. Regular visits were made to record various phenological events of the inflorescences and flowers, and the behavioural patterns of insects at the flowers. Representative samples of insects were collected, and identified by the courtesy of the CAB Identification Service, Commonwealth Institute of Entomology, London.

To find the male to female flower ratio, 10 inflorescences chosen randomly while the flowers were still in bud, were followed until they ceased to flower recording every day the male and female flowers that opened. Daily anthesis of female and male flowers, and anther dehiscence were recorded using tagged inflorescences made free of already opened flowers. Pollen output of an anther was determined squashing mature and undehisced anther in a droplet of lactophenol aniline-blue and spreading the entire mass into a narrow band, and then scanning the total area for pollen using the light microscope. Terminal velocity of pollen was calculated using Stokes's formula. Pollen fall pictures were obtained on microscope slides kept beneath the dehisced anthers to which a gentle touch was applied to know of the clumping of pollen. *In vitro* studies with culture media* that gave the highest germination, helped to assess the pollen viability period. Stigma receptivity was based on the fruit set ability after successive hand-pollinations. To determine pollen depletion rates, a sample of flowers anthesed in unison was marked, and from this, batches of anthers were examined periodically for the pollen remaining in the anther sacs; the difference between two successive counts gave the pollen amount depleted for that interval. Circadian patterns of pollen incidence levels of *Phyllanthus pinnatus* were studied by operating Hirst spore trap (Hirst 1952), and those of *Croton bonplandianum* by employing sticky vertical cylinders of 0.53 cm diameter. Pollen deposition on the stigmas was determined bagging 100 unreceptive flowers, and exposing them for the desired interval in batches after they attained maturity. The exposed stigmas were pressed in between two microscope slides with a droplet of lactophenol aniline-blue and observed under light microscope for pollen. The activity of insects was assessed by scoring the visits they made to a selected group of flowers (male or female flower) for a half-an-hour in every hour during the period the insects were active. The resulting data also enabled to calculate the average time spent by a particular insect at the flower. Female flowers in bud enclosed in butter paper bags were used to test for apomixis, geitonogamy, and xenogamy. After leaving sufficient time for fruit formation, the fruits developed and the seeds formed were scored. To discern the relative role of wind and insects as pollinating agents, batches of plants were kept in insect proof cages and the fruit and seed set rates were compared with the naturally occurring rates.

Results and Discussion

Floral Dynamics of Croton bonplandianum

Inflorescence: 4–10 mm long, monoecious with male and female flowers positioned on the same axis, the former distally and the latter proximally (figure 1a), the two zones overlapping very rarely. The female flowers

**C. bonplandianum:* Brewbaker and Kwack's Medium in liquid form (5ml) with 0.5 to 1.0g sucrose; *P. pinnatus*: Agar 0.5 g+Gelatin 0.5 g+2 g sucrose in 35 ml of tap water (semisolid medium)

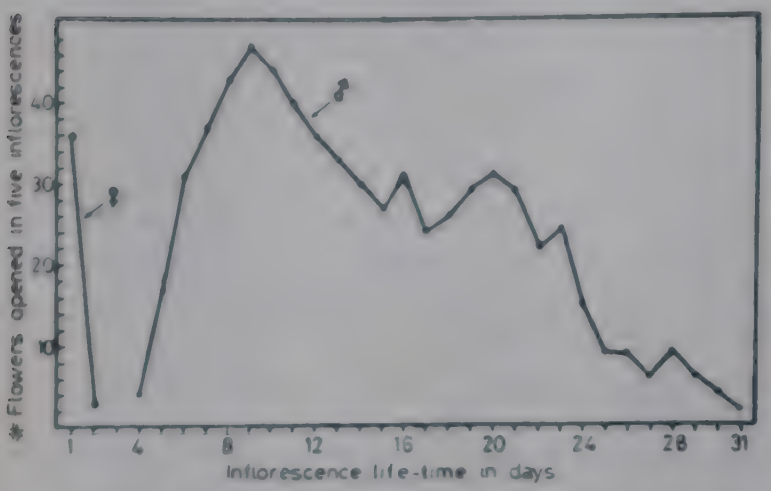

Figure 2 Anthesis rate of female and male flowers in total five inflorescence of *Croton bonplandianum* as a function of time (days)

vary from 5–11 per inflorescence, the male ones from 101–185 (133 ± 32). The female phase lasting two days starts first, while the male phase begins after the cessation of the female phase, and lasts 21–31 days (figure 2). The male flowers are produced in fascicles of 4, rarely 3, at each point and each one matures on a different day.

Staminate flowers

Flower morphology: Flowers pedicillate (2 mm long), aromatic, white, whitish yellow when pollen is present in the anther sacs. Sepals 5, green, 1 mm wide and 1.5 mm long. Petals 5, 0.5 mm wide and 2 mm long. Floral receptacle villose with five red glands located at the base. Stamens 16, 15 in three whorls and one in the centre of the flower (figure 1b); in some cases stamens 15 only, the central one being absent. Filaments glabrous, white, 1.5 to 1.75 mm long; anthers 2-celled dehiscing longitudinally. Pistillode absent.

Anthesis and anther dehiscence: The male flowers begin opening from 1500–1600 hr and are fully open by 1600–1700 hr. During the process of anthesis, the centrally located anthers are exposed first. By the time the flower is fully open, all the other stamens also are exposed. Anthers dehisce immediately after exposure to the environment prevailing on normal days. However, rain occurring at the time of anthesis causes delay in anthesis and subsequent anther dehiscence (figure 3). The flowers are ephemeral and drop off the inflorescence axis after 26 hr of their anthesis.

Pollen characters: There are 3650 ± 700 (R 2560–4300) pollen grains produced per flower. Grains are yellow, slightly sticky, spheroidal, inaperturate, dimorphic: 48 ± 2.19 (R 44–51) and 25 ± 2 (R 22–27) μm, the latter constitutes 17% of the total pollen produced. The calculated speed of fall is 8.7 cm/sec. Pollen fall pictures showed that 50% of the grains fall as single ones from the freshly dehisced anthers, while 60% fall as single ones from the one-day-old anthers. The smaller grains are inviable as they fail to take stain. The larger grains put forth germ tubes within half an hour after placing in the medium. The same sample of pollen remained viable for 168 hr. Freshly collected pollen gave 78% germination with 250 μm long germ tubes. Pollen stored for 120 hr of anther dehiscence showed 50% germination.

Pistillate flowers

Flower morphology: Flowers inconspicuous, provided with a 0.5 to 1.0 mm long pedicel. A large greenish and shiny gland is situated

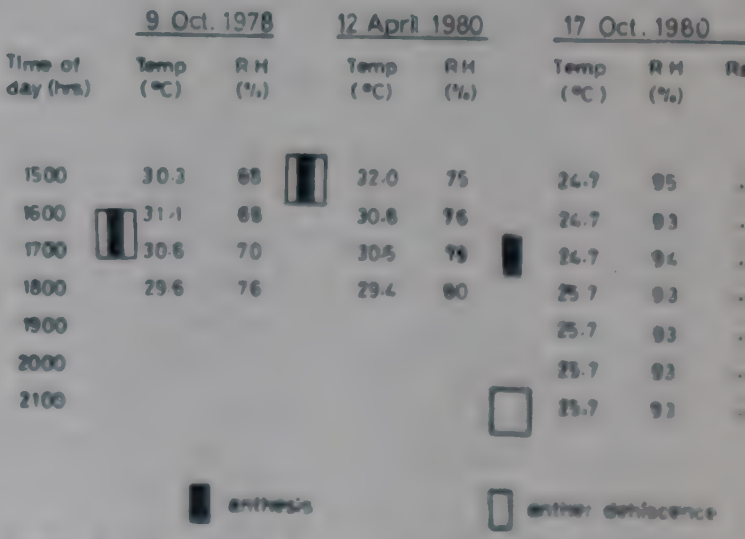

Figure 3 The timing of anthesis and anther dehiscence in *Croton bonplandianum* in different environmental regimes

Figure 1 a–j The floral features and the floral visitors of *Croton bonplandianum*. **a,** Inflorescence in female phase (left) and male phase (right); **b,** Male flower with dehisced anthers; **c,** Gland at the base of female flower; **d,** Female flower with the characteristic forked stigmas; **e,** *Apis cerana indica* collecting pollen at the male flowers; **f,** *Camponotus* sp. collecting the secretions at the basal gland of the female flower; **g,** *Chrysocoris patricius* sucking the juice from fruit; *Camponotus* sp. is also seen; **h,** *Culex* sp. sucking at the male flower and; **i & j,** *Sarcophaga* sp. licking at the female and male flower respectively

at the base of the flower (figure 1c). Calyx lobes five, free, 1.5 to 1.75 mm long and 0.5 to 0.75 mm wide, green. Corolla absent. Disc consists of five red glands. Ovary densely covered with the stellate hairs; three carpellate, each with one ovule. Styles three, free, once bifid sometimes twice; stigmatic lobes slender, 2 mm in length (figure 1d).

Maturation of the pistillate flower: The stigmatic portion becomes visible in the morning hours. At that time they look pale green, inrolled, undeveloped and unreceptive. With the advancement of time, uncoiling of the stigmatic lobes, their expansion, change of colour from pale green to white take place for about 11 hr. Further, traces of sticky secretions are visible on the stigmas. After that time, the stigma appear white and well stretched. This phase lasts for another 48 hrs after which time the stigmatic lobes turn black and appear dry. From the controlled pollinations, it is found that the stigmas remain receptive for 59 hr of anthesis yielding 100% fruit set and for 83 hr yielding 70% fruit set. Thereafter fruit set ability decreased to zero level by 107th hr.

Pollen depletion from anthers vs. pollen deposition onto the stigmas and rod impaction traps

About 46% of the pollen grains produced was removed by the honey bees (*Apis cerana indica* and *A. florea*) during 1600-1800 hr. from the dehisced anthers. About 8% of the total pollen was dislodged during night-time, most probably due to the activity of Dipterans and Thysanopterans. From 0600 to 1200 hr about 27.7% of the total pollen was wafted away mostly by the wind; the Dipterans and Hymenopterans also involved in pollen removal. From 1200 to 1800 hr 13.5% of pollen was dislodged. By the time the male flowers were shed, the anthers contained 5% of the total pollen output (table 1).

Deposition of pollen on the stigmas occurred during daytime and two periods of higher reception: 0800–1200 hr and 1600–1800 hr, could be recognised (table 1). This pattern was reflected in the temporal pattern of pollen deposition onto the rod impaction traps also (figure 4). However, the catching efficiency of the stigmas exceeded several times that of the traps. The average number of pollen

Table 1 *Temporal variation in pollen depletion from anthers vs. pollen deposition onto stigmas of* Croton bonplandianum *and onto rod impaction traps*

Time (hr)	Mean No. of pollen depleted/ flower	Rate of pollen depletion (%)	Mean No. of pollen deposited onto stigma (3.5 mm²)	Rate of pollen deposition (%)	Total pollen deposited on trap (324 mm²)	Rate of pollen deposition (%)
1800	1672	45.8	7	15.5	14	17.9
0600	292	8.0	0	0.0	0	0.0
0800	447	12.3	2	4.4	4	5.1
1000	329	9.0	8	17.8	13	16.7
1200	232	6.4	10	22.2	22	28.2
1400	199	5.5	4	8.9	3	3.8
1600	183	5.0	5	11.1	4	5.1
1800	110	3.0	9	20.0	16	20.5

n = 10 n = 20

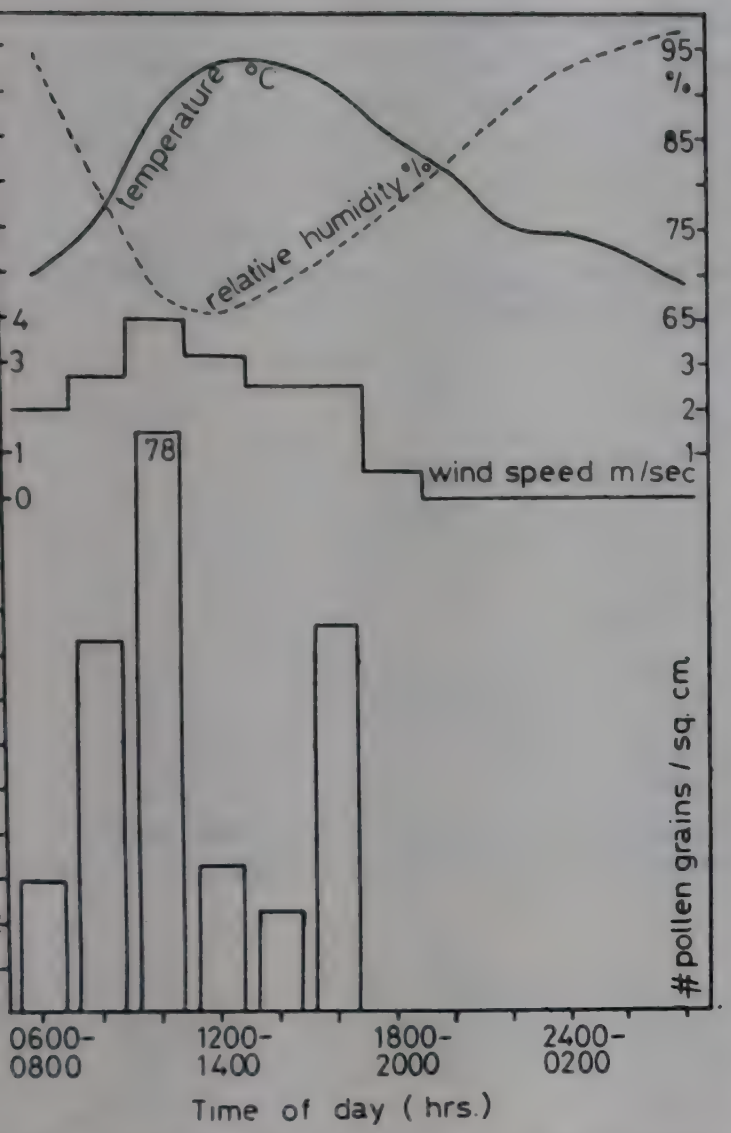

Figure 4 Two-hourly variations in the airborne pollen of *Croton bonplandianum* related to the prevailing weather

grains deposited per sq mm of stigmatic surface was 12.86, and that on rod impactor was 0.24.

Pollen-ovule ratio: Nearly 20570 pollen grains are produced against a single ovule. Out of these only 0.145% reached the stigma.

Insect Activity Dynamics

A total of 18 insect species from six orders was caught during their visits to the flowers of *C. bonplandianum*; their latin names are furnished in table 2 along with the forage type on which they feed.

Intrafloral behaviour of various insect visitors: Among the Hymenopterans, the honey bees (*Apis cerana indica* and *A. florea*) were more frequent. The activity of the *Apis* species was directed towards collecting pollen (figure 1e), consequently their visits were confined to the male flowers only. Their activity was rather brisk during 1600–1800 hr and was normal during 0630–1000 hr.

The ants mostly of *Camponotus* sp. were less frequent. They moved along the inflorescence axis licking the glands present at the base of female flowers and fruits (figure 1f). They rarely came into contact with either male flowers or the females in receptive condition.

The wasps (*Ropalidia spatulata*) were of sporadic occurrence and that too on male flowers only. They did not forage on the flowers; their visits were directed towards obtaining their prey that included the honey bees visiting the male flowers.

Aulacophora lewisii of Coleoptera visited the male flowers only and fed on the delicate tissues of the basal villose receptacle. They never visited the female flowers.

Two species of Heteroptera made visits to the developing fruits. They were seen piercing their proboscis into the fruit wall to obtain the juice (figure 1g). Although foraged on the fruit, they landed first on the distal region of the inflorescence and walked down along the inflorescence axis. While doing so, they got dusted with pollen. However, their role in pollen transfer was unlikely as their visits were solely confined to the developing fruits.

Eight species of Diptera were found visiting the flowers. Their visits were directed toward licking the villose receptacle region of male flowers and the basal gland, the ovary and sticky stigmas of the female flower (figure 1 h–j) They were active during 0630–1000 hr and 0200–0530 hr. They spent more time on the flowers compared to other visitors, and their visits were irregular. The frequency of their visits to male and female flowers was not uniform; the visits to male flowers were approximately four times greater. The insect

Table 2 *Floral visitors on* Croton bonplandianum

Insect species	Forage type
THYSANOPTERA	
THRIPIDAE	
Thrips hawaiitensis Morgan	Traces of secretion of male flowers (receptacle region) & female flowers (basal gland and ovary surface)
HETEROPTERA	
PENTATOMIDAE	
Chrysocoris patricius Westw.	Juice from fruits
C. purpureus Westw.	Juice from fruits
COLEOPTERA	
CHRYSOMELIDAE	
Aulacophora lewisii Baly	Villose receptacle of male flowers
DIPTERA	
MUSCIDAE	
Musca crassirostris Stein	Traces of secretion of male flowers (receptacle region) & female flowers (basal gland and ovary surface)
CALLIPHORIDAE	
Chrysomya rufifacies Macquart	-do-
C. megacephala F.	-do-
Stomorhina discolor F.	-do-
Rhyncomya viridaurea Wiedemann	-do-
SARCOPHAGIDAE	
Sarcophaga orchidea Boettcher	-do-
Sarcophaga sp.	-do-
CULICINAE	
Culex sp.	-do-
HYMENOPTERA	
APIDAE	
Apis cerana indica F.	Pollen
A. florea F.	-do-
VESPIDAE	
Ropalidia spatulata Vecht	Predates on bees
FORMICIDAE	
Camponotus sericeus F.	Secretions of basal gland of female flowers
Camponotus sp.	-do-

after alighting on the inflorescence in female stage walked down the inflorescence to rest on the female flower where it licked on the surface of the ovary and the basal green shiny gland. Thus foraging, it contacted the receptive stigmas. The female flowers receiving such visits showed pollen grains ranging from 60–106 on their stigmas. When the female flowers on different inflorescences from different plants in a population were analysed for pollen deposition on the stigmas, only 15% of the flowers showed such heavy deposition of pollen, thereby suggesting that the Dipterans could cover 15% of the female flowers in a population and, essentially their visits were not regular.

Members of Thysanoptera were very common in both male and female flowers. They may carry out some pollination when the individual plants are in both male and female phases.

Modes of Reproduction

Apomixis is totally absent. Pollination and subsequent fertilization can be effected by both xeno- and geitono-gamous pollen (table 3).

Floral Dynamics of Phyllanthus pinnatus

Inflorescence: The plants shed leaves twice or thrice in a year, each time following a dry spell. After defoliation, several brown dormant flower buds become evident on the stems at almost every node (figure 5a). With the onset of rains after the dry spell, these quiescent buds get activated.

The male flowers (13–58) are produced in dense axillary fascicles at the rate of one fascicle per node on the stem which is bare of leaves (figure 5b). In the very brief period of flowering, most of the flowers (70%) in a population of plants anthese on a single day and the remainder on the second (25%) and on third (5%) days (table 4). The number of flowers produced in each of the flowering cycles was not the same. Of the total of flowers to be produced in the season, 39% was produced in the first cycle, 28% in the second cycle and 33% in the third cycle (table 5).

Like male flowers, the female ones too are produced in axillary fascicles with 5–10 flowers in each fascicle (figure 5c). Unlike the male ones, female flowers matured in two days only; most of them (90%) in a single

Table 3 *Fruit and seed set, and fecundity in different modes of reproduction in* Croton bonpalndianum

Treatment	No. of flowers observed	No. of flowers set fruit	Fruit set (%)	Seed set (%)	Fecundity (%)
Apomixis	50	0	0	0	0
Geitonogamy	50	50	100.0	98.00	98.00
Xenogamy	50	50	100.0	97.40	97.40
Plants covered with thick cloth					
(*a*) Plants with male & female flowers	85	10	11.0	76.59	9.02
(*b*) Plants with female flowers only	70	0	0	0	0
Plants covered with mosquito net					
(*a*) Plants with male & female flowers	180	136	75.5	78.43	59.26
(*b*) Plants with female flowers only	110	72	65.0	71.74	46.97
Open pollination	195	156	80.0	96.70	77.40

Table 4 *Rate of flowering on successive days of the first blooming cycle in* Phyllanthus pinnatus

Blooming day	No. of male flowers produced	Rate of production (%)	No. of female flowers produced	Rate of production (%)
1st	343	70.00	102	90.0
2nd	124	25.00	11	10.0
3rd	25	5.00	0	0.0

n = 15 inflorescences

Table 5 *Rate of flowering in different blooming cycles in* Phyllanthus pinnatus

Blooming cycles S.No.	No. of male flowers produced	Rate of production (%)	No. of female flowers	Rate of production (%)
1st	492	38.60	113	42.00
2nd	360	28.25	72	26.76
3rd	422	33.12	84	31.22

n = 15 inflorescences

day and the remainder the following day (table 4). The proportion of flowers produced in different cycles were: first cycle 42%; second cycle 27%, and third cycle 31% (table 5). The number of female cycles depends on whether or not successful fruiting results in a large proportion of the flowers in the first cycle. If there is good fruiting, the ensuing cycles will be very feeble or may even completely be eliminated.

Staminate flowers

Flower morphology: Flowers pale-yellow-green, 4–5 mm in diameter provided with slender pedicels of varying (1 to 20 mm) lengths (figure 5b). Sepals 6, rarely 7, arranged in two whorls, obovate, 1.5 to 2.0 mm long, more reduced than in female flowers. Petals absent. Disc flattened with six depressed lobes alternating with the sepals. Stamens 6, rarely 7, erect, filaments slender, free, uneven in length (1.5–2 mm). Anthers small, 2-celled and dehisce by longitudinal slits.

Anthesis and anther dehiscence: The process of flower opening is very slow. It starts from 1700 hr and is complete by 0600 hr the next day. The flowers are ephemeral and drop off the next day morning or evening.

The timing of anther dehiscence was mostly controlled by the prevailing weather conditions (table 6). Air temperatures above 28°C and RH below 85% were found to be conducive for anther dehiscence. On cloudy days with low temperature and higher RH the process was postponed until the setting in of optimum conditions. On normal days, anther dehiscence occurred between 0700 and 1000 hr. It is synchronous in about 70% and asynchronous in 30% of the flowers.

Pollen characters: A flower produces 5680 ± 1270 (R 4365–6539) pollen grains. The grains are slightly sticky, spheroidal, tricolporate, 24 ± 2 μm (R 22–26) in diameter with a fall velocity of 1.44 cm/sec.

Of the pollen collected immediately after anther dehiscence, 96.5% germinated with the maximum tube length of 1000–1100 μm. The same sample of pollen gave 50% germination after 72 hr, and none could germinate after 120 hr of storage.

Pistillate flowers

Flower morphology: Flowers green, 5 mm in diameter, provided with slender pedicels 2.75 to 4 cm long (figure 5c). Sepals six, (three larger and three smaller), arranged in two separate whorls. Petals absent. Disc flattened with six depressed lobes alternating with the calyx lobes. Ovary 3-celled, each cell with two ovules. Styles three, 1 mm long and deeply divided into two sharp recurved segments. Staminodes sometimes present 1–3 in number.

Flower maturation: The flowers anthese by 1800 hr; thereafter enlargement of stigma takes place until 0600 hr the following day by

Figure 5 a–g The floral features and the floral visitors of *Phyllanthus pinnatus*; **a,** Branch showing dormant floral buds; **b,** Branch with clusters of male flowers in anthesis; **c,** Branch with clusters of female flowers in anthesis; **d,** *Apis florea* collecting pollen at the male flowers; **e, & f,** *Ca nponotus* sp. collecting the nectar traces at the male and the female flowers respectively and; **g,** *Rhyncomya viridaurea* collecting nectar traces at the male flower

which time the stigmas become turgid and receptive. This condition of the stigma lasts for 34 hr. After 48 hr, the stigmas turn black thereby becoming unreceptive. Controlled pollinations indicated that the stigmas remained receptive for 24 hr. yielding 100% fruit set. With further passage of time, fruit set ability on pollination decreased to zero levels by the 48th hr.

Pollen depletion from the anthers vs pollen deposition onto the stigmas: There is close parallelism between pollen depletion and pollen deposition (table 7). Higher rates of pollen depletion from the anthers and pollen deposition onto the stigmas occurred between 0800 and 1000 hr. Thereafter, depletion sharply declined and reached zero levels by 1800 hr; the rate of deposition onto the stigmas diminished and attained zero levels by 1400 hr. The Hirst trap data (figure 6) show that the occurrence in air of the pollen grains is solely diurnal, mostly confining to the period between 0800 and 1600 hr. Peak concentrations can occur between 0900 and 1000 hr. This rhythmicity of airborne pollen concentrations is identical to the pattern of pollen depletion and pollen deposition.

The period of effective pollination would therefore be during 0800 and 1200 hr, 86%

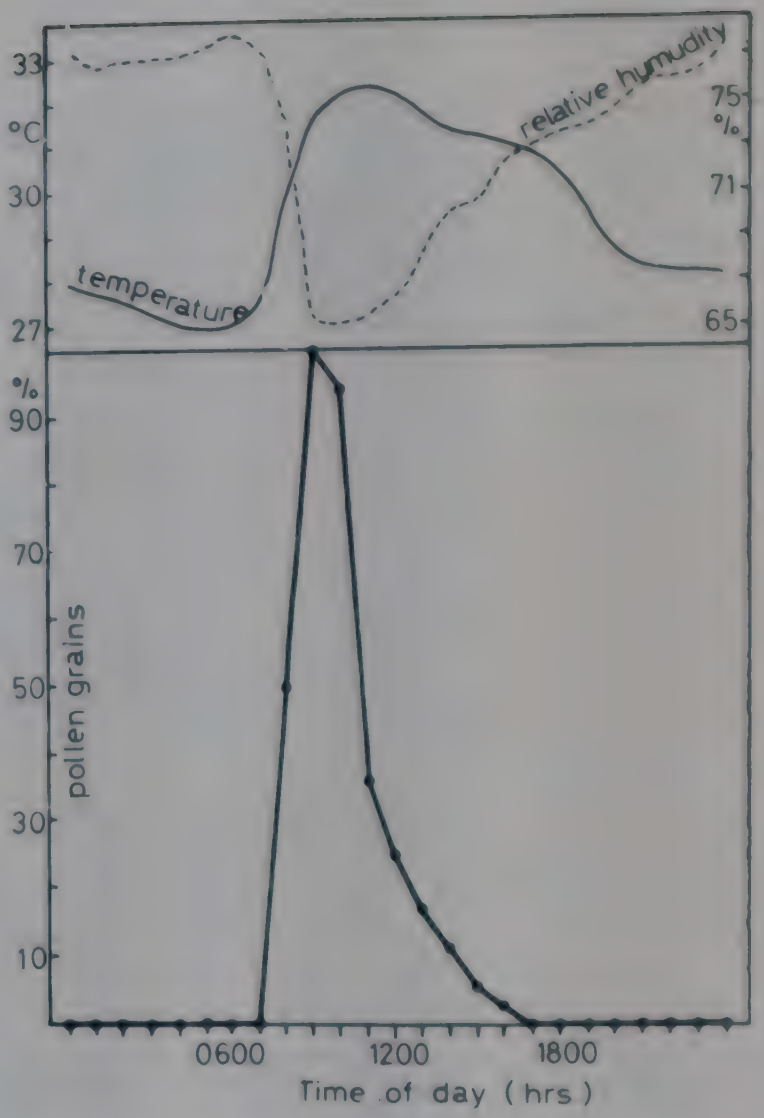

Figure 6 Hourly variations in the air-borne pollen of *Phyllanthus pinnatus* related to the prevailing weather. The data represented are the percentage of peak arithmatic mean concentration

Table 6 *Rate of anther dehiscence in* Phyllanthus pinnatus *on three different occasions in relation to the prevailing weather*

Time (hr)	2-3-1979			18-4-1979			19-4-1979		
	T	RH	DA	T	RH	DA	T	RH	DA
0600	26.8	84	0	27.0	85	0	28.0	85	0
0700	27.9	84	0	27.2	85	0	29.5	80	14
0800	30.5	80	38	28.5	79	0	32.0	67	50
0900	30.7	78	41	31.5	70	60	33.0	64	11
1000	30.6	76	25	32.0	65	25	35.5	60	33
1100	30.8	75	0	32.2	65	23	35.5	60	0
1200	31.1	75	0	32.2	64	0	35.0	60	0

T, Temperature (°C); *RH*, Relative humidity (%); *DA*, No. of dehisced anthers

Table 7 *Temporal variation in pollen depletion from anthers vs. pollen deposition onto stigmas of* Phyllanthus pinnatus

Time (hr)	Mean No. of pollen depleted/ flower	Rate of pollen depletion (%)	Mean No. of pollen deposited onto stigma	Rate of pollen deposition (%)
0800	0	0.0	0	0.0
1000	3862	68.0	8	61.5
1200	1022	18.0	3	23.1
1400	398	7.0	2	15.4
1600	142	2.5	0	0.0
1800	34	0.6	0	0.0

n=10 flowers

of the total pollen output is being removed and 85% of total pollen deposition is released during that period.

Pollen-ovule ratio: Nearly 15000 pollen grains are produced against a single ovule. Out of these, 0.047% reached the stigma.

Insect Activity Dynamics

The different insect species caught at the flowers are listed in table 8. Seven of the 11 species collected, belong to the Hymenoptera and the remaining four to the Diptera.

Among the Hymenopterans, the honeybees restricted their visits to male flowers and collected pollen (figure 5d). They were active from the time of anther dehiscence (0700–1000 hr) until 1400 hr and henceforth disappeared. The ants numerically preponderated; out of the 227 insect visits recorded on one occasion, ant visits alone constituted 79%. They visited the flowers during 0600–1800 hr, though in varying frequency, exhibited no apparent bias to the flower sex, and concentrated on taking the traces of nectar (figure 5 e & f). Their activity increased rapidly commensurate with the increase in air temperature until 1000 hr, when the recorded temperature was 32.5°C. With further rise in air temperature, the activity fell to minimal levels by 1400 hr and thereafter the temperature tended to decrease with the concomitant rise in activity until 1700 hr and then declined. During their visits to male inflorescences, they got dusted with pollen grains all over their body, the number of grains varied between 9.29 ($\bar{X}$ = 18.7± 6.8). However, when the ants crawl onto the females, most of the pollen adhered to the ventral surface is liable to be lost whereas the pollen grains of other regions are not affected (n=10; $\bar{X}$ = 7.6; SD = 3.83; R 3-15). Moving on the female flowers, the ants touch the recurved stigmas with their lateral and dorsal portions of the body. They mostly made repeated visits to the same flowers.

The different Dipterans recorded, made visits to male and female flowers and were busy licking the secretions at the disc of both kinds of flowers (figure 5g). Their pattern of diurnal activity is almost identical with that of ants. Fly body washings revealed considerable number of pollen grains (n=10; $\bar{X}$=44.8 ± 16.75; R 23–64 on male flower; n=10 $\bar{X}$ = 38.5 ± 13.8; R 17–53 on female flower). It is found that the female flowers receiving the fly visits will have relatively higher amounts of pollen deposited on their stigmas. When randomly selected stigmas in a population of plants with both male and female flowers spaced closely were screened, only 32% of them had ⩾30 pollen grains. Obviously. the population of flies was not adequate to cover all the flowers in the populations studied.

Modes of Reproduction

Apomixis is totally absent. As the plants are dioecious, xenogamy is a must.

Pollination

In both the species, the floral features such as the inconspicuous flowers, production of

Table 8 *Floral visitors on* Phyllanthus pinnatus

Insect species	Male plant	Female plant	Purpose of visit
HYMENOPTERA			
Apidae			
Apis cerana indica F.	+	−	Pollen collection
Apis florea F.	+	−	-do-
Apis sp.	+	−	-do-
Anthophoridae			
Ceratina sp.	+	−	-do-
Halictidae			
Pseudopis oxybeloides Smith	+	−	-do-
Formicidae			
Camponotus sp.	+	+	Traces of secretion at disc region of both male & female flowers
Camponotus sariceus F.	+	+	-do-
DIPTERA			
Sarcophagidae			
Sarcophaga sp.	+	+	-do-
Muscidae			
Musca crassirostris Stein	+	+	-do-
Musca pattoni Austen	+	+	-do-
Calliphoridae			
Rhyncomya viridaurea Wiedemann	+	+	-do-

Table 9 *Fecundity in* Phyllanthus pinnatus *as influenced by pollinations mediated by different agents*

Type of agent pollinating	Pollen loads of stigmas	No. of flowers observed	No. of flowers set fruits	Fruit set (%)	Seed set (%)	Fecundity (%)
Female plants in proximity of male ones						
(*a*) Plants left open to wind but excluded from insects	4.25±2.44 (n=50)	485	310	64.0	66.6	42.6
(*b*) Plants left open to both wind and insects	24±14 (n=50)	500	490	98.0	88.4	86.6
Female plants spaced 20 m from male ones						
(*a*) Plants left open to wind but excluded from insects	1.69±1.43 (n=25)	500	238	47.6	27.0	12.9
(*b*) Plants left open to both wind and insects	2.24±3 (n=25)	500	250	50.0	28.3	14.2

rather good amounts of pollen grains which become airborne (figures 4 & 6) speak in favour of wind as the agent of pollination. The presence of traces of nectar and the frequent visits of certain insects to these nectars, and of some to collect pollen, suggest that insects also take part in accomplishing pollination.

Then what exactly are the relative contributions of these agents to the pollination in these two species? This was ascertained from the fruit set and fecundity values obtained after controlled and natural pollinations. In *P. pinnatus* populations with closely spaced male and female plants, fruit set to the tune of 64% was realised due to wind pollination alone (table 9). The rate of fruit set has been enhanced by 34% when the insects were also allowed to take part in the act of pollination along with the wind. By contrast, in populations with the female plants farther apart from the pollen sources, the wind could bring about substantial fruit set (47.6%), whereas the addition by the insects is negligible and is only 2.4%. In *C. bonplandianum*, when plants were kept off from insect visitation, fruit set to the tune of 75.5% was realised as against the natural fruit set rate of 80% (table 3); obviously the increased fruit set in open pollination is accountable for the activity of thrips and Dipterans. In other words, it can be expected that 94.5% of the natural fruit set results from pollination mediated by wind alone, whereas the other 5.5% from insect activity.

Based on fruit set rates it is clear, that the wind has proved superior as pollinator over the insects. However, in terms of fecundity the role of insects assumed some importance. In the absence of insect visitation, the wind could bring about 43% fecundity in *P. pinnatus* and 59% in *C. bonplandianum*. As opposed to this, the combined action of wind and insects resulted in 86% fecundity in *P. pinnatus* and 77% in *C. bonplandianum*.

Fecundity is a function of the number of pollen grains deposited on the stigmas. Then the maximised fecundity under the combined activity of insects and wind should result from increased pollen deposition. This has become true. The observations for the extent of pollen deposition due to wind alone, and wind and insects together showed respectively 10 and 62 pollen grains per stigma in *P. pinnatus*.

Though the two taxa rely mainly on wind for pollination, they differ in the time of pollen presentation, and also in pollen characters. It is necessary that in dioecious plants the pollen grains have to be transported to relatively longer distances. This is possible when the grains are liberated at times when there will be optimum turbulence. In this part of the globe, such conditions begin to exist after sunrise when there is increasing temperature and decreasing RH. In *P. pinnatus* pollen grain size (25 μm) and low terminal velocity (1.44 cm/sec) are admirably suited for turbulent wind transport. In monoecious plants, that too exhibiting plasticity in breeding system, there is not that much need for the pollen to be carried to longer distances. In circumstances where the plants do not occur in close proximity, liberation of pollen during turbulent conditions results in gamete wastage. In *C. bonplandianum*, the larger size of the pollen grains (48 μm) with high fall velocity (8.7 cm/sec) is rather ill suited for dissemination to longer distances. Rather it is meant for short distance transport. The pollen grain characters coupled with the timing of pollen presentation at times when conditions damp out turbulence, as is also the case with several Poaceae members (Reddi 1982, Subba Reddi & Reddi 1984), are geared to achieve pollen economy and to maximise reproduction through geitonogamy. Of course, the chances for outcrossing (xenogamy) are not entirely ruled out in this taxon because the inflorescences are protogynous, and during

the forenoon hours there may be taking place some xenogamous pollination mediated by the turbulent wind. Outcrossing may also be mediated by insects especially the Dipterans.

Thus, the two taxa of the present study, together with *Cicca acida* and *Emblica officinalis* (Reddi 1981, Subba Reddi & Reddi 1984), clearly demonstrate that the selective pressures for the timing of pollen presentation operating in dioecious taxa are different from those operating in monoecious taxa. Furthermore, the fact that these two taxa are pollinated mainly by wind, and *C. acida*, *E. officinalis* (Reddi 1981, Subba Reddi & Reddi 1984), *Madhuca indica* (Subba Reddi 1976), *Mimusops elengi* (Subba Reddi & Janaki Bai 1981), and *Trophis involucrata* (Bawa & Crisp 1980) exclusively by wind, points to the refutal of the general notion that wind pollination is absent or rare among trees and shrubs in the tropics.

Acknowledgements

The authors thank Prof L W Macior University of Akron, Ohio, USA for his critical comments, EUBR is thankful to the Council of Scientific and Industrial Research for financial help.

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Proc. Indian natn. Sci. Acad. B51 No. 4 pp 483-489 (1985

Anthoceros bharadwajii—A New Species from India*

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(Received 17 December 1984; after revision 6 June 1985)

Anthoceros bharadwajii sp. nov. is described primarily based on sporoderm characteristics which are compared with those of *A. erectus*. Spores maintain stable differentiating characteristics being reticuloid, pseudolamellate to lamellate in the former and reticulate in the latter. Both taxa show maximum differentiation in Kerala.

Key Words: Hornworts, Anthocerotaceae, *Anthoceros bharadwajii* sp. nov.

Introduction

Anthoceros erectus described by Kashyap (1915, see also 1929) from Western Himalayas (Kumaon, Mussoorie, Kulu, Manali) and South India (Madras, Travancore), exists in nature in various morphoforms. The taxon is characterized by thalli raised on a cylindrical stalk expanding above, often becoming fan-shaped and having deeply lobed margins. The morphological details of the plants were provided by Mehra and Handoo (1953). Bharadwaj (1960) showed that a characteristic reticulate sculpturing occurs on proximal and distal faces of the spores and there is an unsculptured stripe along both the sides of laesura in *A. erectus*. He described *A.* cf. *erectus* from Pachmarhi (Madhya Pradesh) which resembles *A. erectus* in thallus morphology but differs in exhibiting reticuloid sculpturing on spore surface and comparatively longer elaters. Both maintain stable differentiating features in nature particularly in their sporoderm morphology, which under SEM get clearly resolved. Study of a number of populations from different bryogeographic regions of the country warrant *A.* cf. *erectus* being raised to the status of a new independent taxon.

Materials and Methods

Specimens of *A. erectus* and *A.* cf. *erectus* were collected from different parts of India. The former occurs at Heena (Western Himalayas), Ukhrul (Eastern Himalayas), Kodaikanal, Naduvattam (Tamil Nadu), Periavurrai and Devicolam (Kerala). The latter is relatively more common being luxuriant at Nainital (Western Himalayas), Darjeeling, Ukhrul,

*Contribution from the Department of Botany, University of Lucknow, Lucknow, New Series (Bryophyta) No. 198

Shillong peak, Mao (Eastern Himalayas), Naduvattam (Tamil Nadu), Devicolam, Periavurrai, Murukkady, Peermade, Munnar and Trichur (Kerala). Observations were made from herbarium as well as alcohol-fixed specimens.

Taxonomic Description

Anthoceros bharadwajii sp. nov.*
(plate 1, figures 1–19; plate 2, figures 1, 2)

A. cf. erectus Kash., Bharadwaj
in *J. Indian bot. Soc.* 39 568–592, 1960

Species haec ab *A. erectus* Kash. differt episporio reticuloido-pseudolamellato-lamellato.

Typus: Leg. R. Udar *et* party, prope Trichur, Kerala, India, October, 1982. LWU 5446/82.

Plants dioecious (?), female thalli radially oriented to fan shaped, margin lobed, slight to deeply dissected, spongy, with mucilage chambers in one tier; involucre narrow, 3–5 mm long, air chambers in a single row; spores light to dark brown, spinulate to baculate (often bearing stunted projections), 35–55 μm; proximal and distal faces having projections oriented in reticuloid-pseudolamellate-lamellate pattern; laesura arms straight to arched with an unsculptured stripe 8–17 μm wide bordering on either side, elaters thin-walled, 160–230 μm long, 4-celled or rarely 5-celled, sometimes branched; capsule wall bearing stomata 50×45–62×55 μm.

Holotype: LWU 5446/82. Loc. Trichur (Kerala), Leg. R. Udar & party, Oct. 1982.

Specimens Examined

1. *A. bharadwajii*

Western Himalayas: Nainital, *M.Sc. party* 7-A/80, Sept. 1980 (LWU).

Eastern Himalayas: Nagaland, Mao, *Awasthi & Kumar* 3599-B/79, Oct. 1979 (LWU); Manipur, Ukhrul, *Awasthi & Kumar* 3629-A/79, 3668-A/79, 3698-A/79, 3633/79, Oct. 1979 (LWU); Meghalaya, Shillong peak, *Awasthi & Kumar* 3947/79, 3978/79, Oct. 1979 (LWU).

South India: Kerala, Munnar, *Udar & party* 5838/82, 5890/82, Oct. 1982 (LWU); Kerala, Murukkady, *Udar & party* 6179/82, Oct. 1982 (LWU); Kerala, Peermade, *Udar & party* 6349/82, Oct. 1982 (LWU); Kerala, Periavurrai, *Udar & party* 6023-B/82 Oct. 1982 (LWU); Tamil Nadu, Naduvattam, *Udar & party* 7167/83. Oct. 1983 (LWU).

2. *A. erectus* Kash. (plate 2, figures 3, 4)

Western Himalayas: Pithoragarh, Heena, *Nath* 8055/72 Oct. 1972 (LWU).

Eastern Himalayas: Manipur, Ukhrul, *Awasthi & Kumar* 3675/79 Oct. 1979 (LWU).

South India: Kerala, Periavurrai, *Udar & party* 6023-A/82, Oct. 1982 (LWU); Kerala, Devicolam, *Udar & party* 5949/82, Oct. 1982 (LWU); Tamil Nadu, Kodaikanal (Silver Cascade), *Udar & party* 7490/83, Oct. 1983 (LWU); Tamil Nadu, Naduvattam, *Udar & party* 7167/83 Oct. 1983 (LWU).

Both *A. erectus and A. bharadwajii* possess an unsculptured stripe along laesura of their spores but the basic sculpturing pattern of spore surface is quite different. In an analysis of spore characteristics of different populations of *A. bharadwajii* (table 1) the largest and the smallest range of size was observed in the plants from Kerala, being 52–55 μm in specimens from Trichur and 35–40 μm in specimens from Periavurrai. The surface projections of the spore range from acute to papillate in plants from South India and Western Himalayas (Nainital) to almost perfectly baculate (besides some intermediate conditions) in specimens from Eastern Himalayas (Ukhrul, Shillong, Mao). The sculpturing is pseudolamellate or lamellate to

*Named after Dr D C Bharadwaj

Table 1 *Range of spore characteristics in* A. bharadwajii *and* A. erectus A. bharadwajii *sp. nov*

Locality	Spore size	Distal face			Proximal face	
		Surface sculpturing	Spinulose density	No. of reticulations across the distal face	Nature of triradiate mark	Outline and maximum width of unsculpture stripe
Western Himalayas	42–46 μm	Pseudolamellate	++	conspicuous Reticulations absent	Thin wavy terminating shortly before periphery	Arched, width 8–13 μm in middle
Eastern Himalayas	42–46 μm (Ukhrul, Shillong) 46–50 μm (Mao)	Pseudolamellate to scattered	++	conspicuous Reticulations absent	Thin almost straight to wavy, terminating shortly before periphery	Arched, width 5 μm in middle (Shillong & Mao) 14–17 μm (Ukhrul)
South India	52–55 μm (Trichur) 45–50 μm (Murukkady) 40–47 μm (Munnar, Devicolam) 35–40 μm (Periavurri)	Scattered to pseudolamellate-tending to be reticuloid	+ to +++	conspicuous Reticulations absent	Thin, almost straight to wavy, terminating near periphery	Straight-arched-wider towards periphery width 5 μm & 14 μm (Periavurrai) 9 μm (Munnar) 14 μm (Devicolam & Trichur) 11μm (Murukkady) 8–10μm (Naduvattam)
Western Himalayas	46–50 μm	Reticulate	*A. erectus*	5	Thin almost straight, terminating near periphery	Arched, width 13–17 μm (Heena)
Eastern Himalayas	46–50 μm	Reticulate		4	Thin almost straight, terminating near periphery	Arched, width 13 μm in middle (Ukhrul)

Table 1 *contd.*

South India	50–54 μm (Naduvattam) 46–50 μm (Kodaikanal) 42–60 μm (Devicolam) 40–52 μm (Periavurrai)	Reticulate	5 (Devicolam, Naduvattam) 3–4 (Periavurrai) 4–5 (Kodaikanal)	Thin almost straight terminating near periphery	Arched, wider towards periphery width 8μm (Naduvattam) 9 μm (Devicolam) (Periavurrai) 17μm (Kodaikanal)

dense+++, comparatively less dense++, least dense+

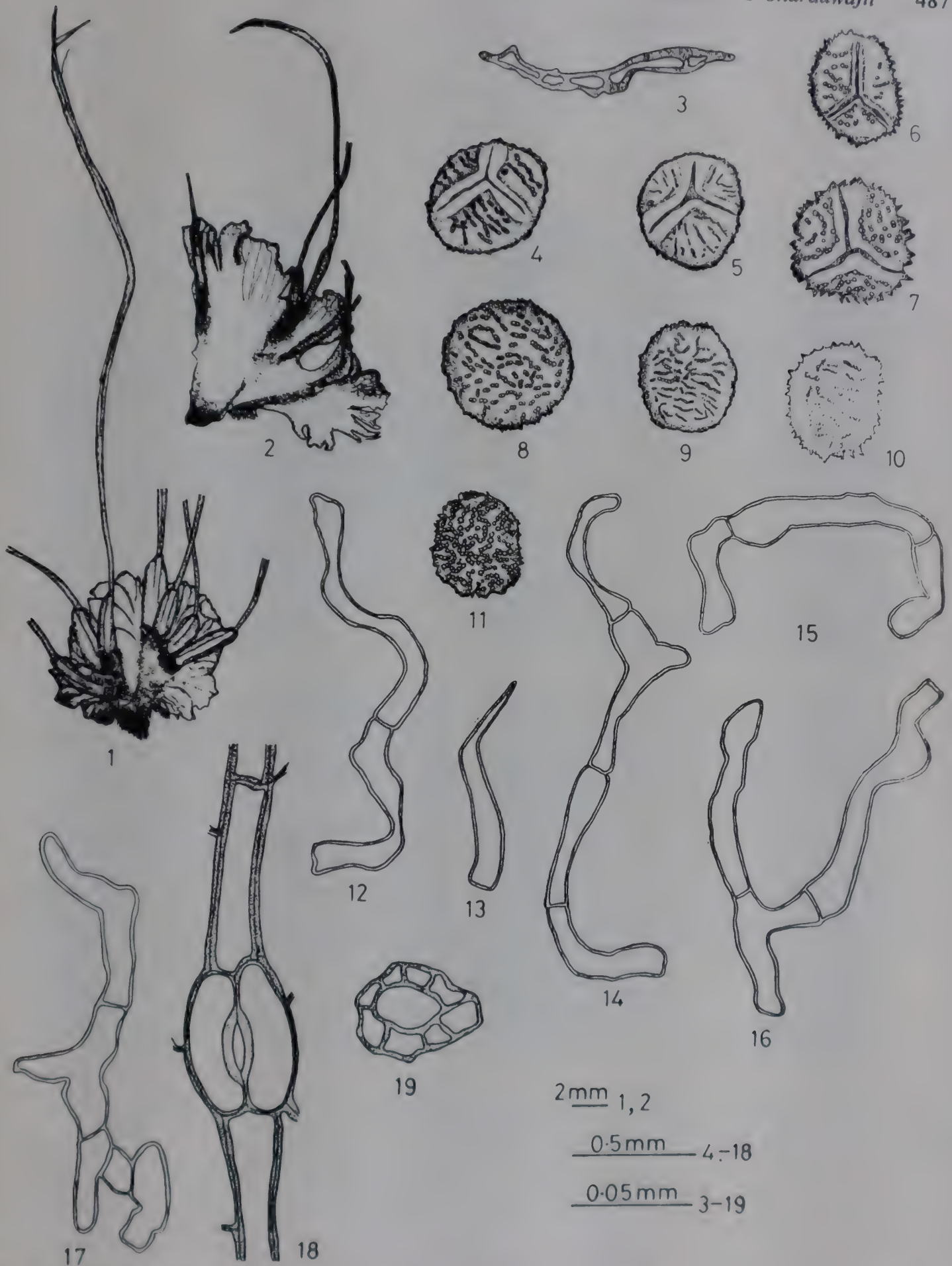

Plate 1, figures 1-19 *Anthoceros bharadwajii*: **1, 2,** Female thallus; **3,** cross section of same; **4-7,** proximal view of spores; **8-11,** distal view of spores; **12-17,** Elaters; **18,** stoma on capsule wall; cross section of involucre (figs 1, 3, 4, 12, 13, 14, 18, 19 based on specimens from Murukkady; 5, 9 from Devicolam; 15, 18 from Munnar; 6, 10 11 from Periavurri; 7, 17, from Trichur)

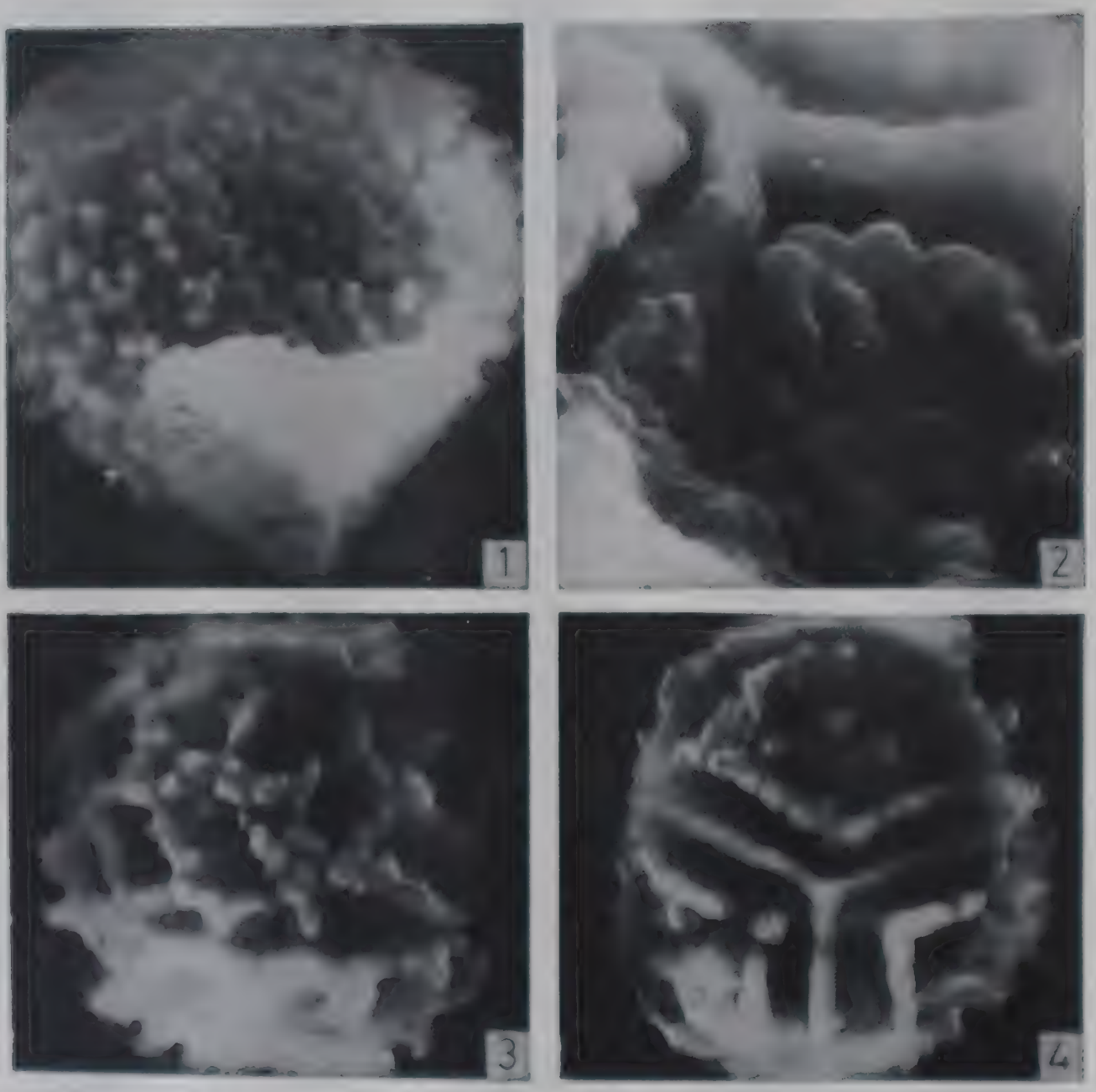

Plate 2, figures 1, 2 *Anthoceros bharadwajii*: **1**, distal view of spore (× 840). **2**, proximal view of spore (× 3500). **3, 4**, *Anthoceros erectus*; **3**, distal view of spore (× 1020); **4**, proximal view of spore (× 930) (figs 1, 2 based on specimens from Ukhrul; 3,4 from Kodaikanal)

reticuloid on the distal as well as on the proximal faces. In addition, a wide range of variation in the orientation and density of surface projections was also observed. There may be an almost beaded lamellate arrangement of surface projections (Devicolam specimens) or diverse irregular orientation leading to a pseudolamellate pattern (Murukkady & Munnar specimens). The density of projections ranges from scattered to extremely dense condition (Periavurrai specimens) with some spores showing intermediate conditions (Trichur, Murukkady, Devicolam & Munnar specimens). An extremely dense orientation and juxtaposition of spines tending to form a reticuloid pattern was observed in plants from Periavurrai. The unsculptured stripe next to laesural arms varies in width from 14 to 17 μm (table 1). Laesural arms may be arched (as in Murukkady and Periavurri Specimens) with a tendency to widen out (Devicolam & Trichur specimens) towards ambi. The laesura is thin, wavy to almost straight and sometimes extending to the ambi (table 1). Under SEM, the spore surface appears densely studded with tuberculate to baculate surface projections. A close juxtaposition of surface projections presents a reticuloid pattern (plate 2, figure 1). On the proximal face a slightly wavy triradiate mark, with reduced projections, is clearly separated from the rest of sculpturing by an unsculptured stripe (plate 2, figure 2). It is significant that in *A. erectus* also (table 1) the largest and the smallest range of spore size was observed in plants from Devicolam (42–60 μm) and Periavurrai (40–52 μm), both from Kerala. However, a slight variation in the spore size (46–50 μm) was also observed in the specimens from Western Himalayas (Heena) and Eastern Himalayas (Ukhrul). The surface sculpturing is reticulate on both the distal and the proximal faces with 3–5 reticulations across the distal face. The unsculptured stripe at the proximal face, running along the laesura, has a maximum width of 17 μm in specimens from Kodaikanal. It may be arched or may widen towards ambi. The laesural arms are almost straight and fall short of ambi (periphery). Under SEM, the spores exhibit bold muri which are studded at top with tuberculate projections (plate 2, figure 3) which gives a tuberculate profile to the spores. The lumina may not be of regular shape. On the proximal face the surface of the laesura shows less conspicuous tuberculate projections (plate 2, figure 4).

Acknowledgements

The authors are thankful to the Department of Science and Technology (SERC), and the University Grants Commission, Government of India, for financial assistance and to the authorities of the Indian Institute of Technology, Kanpur for providing SEM facilities.

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Proc. Indian natn. Sci. Acad. B51 No. 4 pp 490–495 (1985)

Agronomic and Anatomical Characters in Relation to Cold-Tolerance and Grain-Yield in a Maize Composite

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(Received 19 December 1984; after revision 1 November 1985)

One hundred S_1 families of 'Partap' were evaluated in a randomized block design. Data were recorded on agronomic and epidermal characters in all 100 S_1 lines and on anatomical characters in 28 S_1 lines. Significant differences were observed among S_1 families with respect to grain yield, yellowing and other agronomic traits. There was a wide range in the expression of S_1 families with respect to various epidermal and anatomical traits. Cold tolerance was positively and significantly correlated with grain yield, plant height, number of leaves and ear-leaf area. Stomatal size, silica cell size, thickness of lamina, cuticle and upper epidermis and sclerenchyma cell wall and number of sclerenchyma layers showed significant correlation with cold tolerance.

Grain yield was positively and significantly correlated with plant height, number of leaves and ear-leaf area. Stomatal frequency had negative significant and silica cell, microhair and macrohair frequency had positive significant correlations with grain yield. The results of present investigation lead to the conclusion that there was appreciable genotypic variability among S_1 families for cold tolerence and other agronomic, epidermal and anatomical characters. Some of the traits having a close association with cold tolerance may serve as useful parameters in screening germplasm for cold tolerance.

Key Words: *Zea mays* L., S_1 families, Cold tolerance, Grain-yield, Epidermal and anatomical traits

Introduction

In India, maize has traditionally been grown as *kharif* crop from June-July to September-October. However, cultivation during *rabi* season (winter) is gaining importance in states of Bihar, Andhra Pradesh, Karnataka and Tamil Nadu (Anonymous 1980). As high grain yield as 60–80 quintals/ha has oftenly been obtained and yields higher than 80 quintals are also not infrequent.

In view of the high yields of maize being obtained and the possibility of better crop management during winter season, studies

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were initiated at Punjab Agricultural University, Ludhiana in 1977-78 on cultivation of maize during winter (Khehra et al. 1979). Composite 'Partap' performed better than most of other germplasms and has been released for general cultivation in winter season in Punjab in 1983 (Khehra & Dhillon 1983).

Winter in Punjab is severer than other winter maize-growing regions in India. Average temperature during winter may go as low as 10°C and minimum may sometimes touch 0°C. It is generally believed that maize cannot be grown where there is frost incidence or temperature falls below 13°C during growing season (Anonymous 1980). Thus it is evident that winter hardiness will have to be combined with high yield potential and other desirable traits for successful cultivation during winter.

Plant resistance may be manifested in terms of certain morphological and physiological adaptations that allow the plant to tolerate or to overcome the imposed stress (Steponkus 1978). Therefore, a study of the variation and co-variation of morphological and anatomical characters, cold tolerance and grain yield is important in understanding factors contributing to cold tolerance.

The aim of the present investigation was to study cold tolerance and yield in S_1 families of Partap in relation to some agronomic and anatomical characters.

Materials and Methods

One hundred S_1 lines of 'Partap' were grown along with two checks in the research farm of the Department of Plant Breeding, Punjab Agricultural University. The checks included were 'Partap', the parental variety of S_1 lines under evaluation, and local, which has been observed to be cold susceptible and poor yielder in winter. The sowing was done on November 15, 1980 and the design was randomized block design with two replications. Each plot consisted of a row of 5 m length. Row-to-row and plant-to-plant spacings were 60 cm and 25 cm, respectively. Five plants were randomly taken from each plot and data on grain yield per plant (g), yellowing (1–10), stem thickness at 10 cm above ground surface (cm), plant height (cm), number of leaves, ear-leaf area (cm^2) and sheath length (cm) were recorded. Yellowing, an indicator of susceptibility to cold, was recorded on 30 January, 1981, using a scale grading of 1 to 10 where 1 indicated greenness and 10 maximum yellowing. Ear-leaf area was estimated by the formula of Pearce et al. (1975).

Epidermal system of all 100 S_1 families was studied. Ear leaves of five plants on which agronomic data were recorded in the first replication were collected. Long segments (3 to 5 cm) were taken from the middle portion of lamina and immersed in acetone for 3 to 4 days. These segments were treated with 6% H_2O_2 for 5-6 days to decolorize the tissue. Decolorized strips were treated with 80% lactic acid at room temperature for 2–3 days to obtain tissue transparency. The segments were mounted in glycerol jelly. Microscopic examination on the adaxial surface of leaf was made with ordinary microscope. Stomatal frequency (per sq. cm) and the size (μm) of stomata, silica cells, micro- and macro-hairs were recorded.

For studying the internal anatomical characters, 28 S_1 families were sampled from among the groups of families having similar score for yellowing. Five leaflets were randomly selected and preserved in FAA (Formaldehyde, 5 ml + glacial acetic acid 5 ml + 70% ethyl alcohol, 90.0 ml). Cross-sections of the middle portion of the lamina were obtained by using sharp razor blade and then stained with Safranin-fast green combination and mounted in DPX (Johansen 1940). Microscopic measurements of various leaf tissues were made at different locations in five cross-sections of each leaflet using ocular micrometer (μ).

The averages of these observations were calculated. Data on the thickness of lamina in the median part of the blade, the thickness of cuticle and epidermis of adaxial surface, the diameter of spongy parenchyma, the thickness of sclerenchymatous plates extending from midrib vascular bundles on adaxial side, the number of layers in sclerenchymatous plate and the thickness of the wall of sclerenchymatous cells were recorded.

Analysis of variance of randomized complete-block design was carried out to partition the variation due to genotypes, replications and error. To study covariation between two characters, estimates of simple correlation coefficients were obtained. The analyses were carried out following Panse and Sukhatme (1967).

Results and Discussion

There were significant differences among S_1 families of Partap with respect to grain yield, yellowing, plant height, number of leaves, ear-leaf area and sheath length indicating genotypic variability for these characters (table 1). The differences between check varieties, Partap and local were significant for all the traits. The local had lower yield, higher score for cold susceptibility, thinner stem, shorter plants, less number of leaves and ear-leaf area and smaller sheath than Partap (table 2).

In comparison to the differences between local and Partap, cold susceptible and tolerant varieties respectively, the range among the S_1 lines was 2 to 8 times greater for epidermal characters. Among the anantomical characters, the S_1 lines had appreciably greater range than that between local and Partap for diameter of spongy parenchyma cell wall only. This shows a substantial variation among S_1 families of Partap with respect to epidermal characters and the above-mentioned two anatomical characters.

Mean squares due to average performance of S_1 families versus parental variety Partap were significant for grain yield, yellowing and plant height. The grain yield and plant height of S_1 families was lower and yellowing was higher than that of Partap. Such a shift towards poor performance is expected as a result of inbreeding depression.

On the average, the S_1 families had thinner lamina, cuticle and epidermis, sclerenchymatous plates, sclerenchymatous cell wall and lesser number of sclerenchymatous layer. Partap had more silica cells, macrohair and sclerenchymatous layers, longer micro- and macro-hair and thicker lamina, cuticle and epidermis, spongy parenchyma, sclerenchymatous plates and sclerenchymatous cell wall than local.

The correlation coefficients of yellowing and grain yield with other characters in S_1 families of Partap are given in table 3. Yellowing was negatively and significantly correlated with grain yield, plant height, number of leaves and ear-leaf area. This indicates that families which were better for cold tolerance (a lower score for yellowing) had higher grain yield, taller plants, more number of leaves and larger ear-leaf area. Khehra et al. (1981) had evaluated ninety-six diverse genotypes and observed that higher yielding materials generally had less yellowing.

Among the eight epidermal characters, only stomatal size and silica size showed significant negative correlation with yellowing showing that cold tolerant lines had longer stomata and silica cells.

Yellowing had the highest correlation with stomatal size than any other epidermal character. Probably, larger size of stomata results in increased rate of photosynthesis, thus causing increased vigour in leaves. Consequently, there is less yellowing. It may be added that stomatal size is easy to measure and large number of samples can be handled. Thus, it may be used in screening a large germplasm for cold tolerance. Huner et al. (1981) stated that most likely effect of

Table 1 *Analysis of variance of various agronomic characters of S_1 families of Partap*

Source	d.f.	Grain yield	Yellowing	Stem girth	Plant height	No. of leaves	Ear-leaf area	Sheath length
Genotypes	101	1184.05**	514.24**	2.14**	654.47**	1.74**	9983.24**	15.59**
S_1 families	99	988.90**	4.52**	0.89	518.48**	0.47*	9133.25**	3.98**
Checks	1	5625.00**	7.56**	3.90**	1766.01**	3.06**	44073.76**	17.23**
S_1 vs checks	1	246.30	0.38	0.20	514.60**	6.62**	20728.00**	13.09**
Error	101	90.67	0.52	0.83	34.85	0.10	695.17	0.89
S_1 vs Partap	1	4086.01**	2.22**	1.38	180.90**	0.36	2061.00	0.064

*, **Significant at 5 and 1% levels, respectively

Table 2 *Mean and range of performance of S_1 families of Partap and mean performance of checks for various agronomic, epidermal and internal anatomical characters*

Character	Mean	Range		Mean	
		Minimum	Maximum	Local	Partap
AGRONOMIC CHARACTERS					
Grain yield (g/plant)	49.6	10.0	140.0	20.0	95.0
Yellowing (1–10)*	4.31	1.0	8.5	6.0	3.25
Stem girth (cm)	7.92	5.5	9.4	5.95	7.94
Plant height (cm)	92.8	50.9	145.0	60.47	102.4
Number of leaves	16.3	12.4	19.2	14.2	15.9
Ear leaf area (cm^2)	254.2	115.8	462.5	76.6	286.5
Sheath length	13.7	9.8	16.6	9.5	13.8
EPIDERMAL CHARACTERS					
Stomatal size (μm)	44.3	38.6	50.0	43.0	45.7
Stomatal frequency (per cm^2)	8565.3	6400.0	10730.6	7950.0	8425.9
Silica size (μm)	24.0	18.9	29.2	22.9	25.2
Silica cell (and microhair) frequency (per cm^2)	4211.9	2111.1	6312.7	2840.5	4050.8
Microhair size (μm)	51.2	39.3	63.0	44.6	51.7
Macrohair size (μm)	922.2	610.4	1234.0	745.0	910.7
Macrohair frequency (per cm^2)	170.2	125.3	215.1	151.7	182.8
INTERNAL ANATOMICAL CHARACTERS					
Thickness of lamina (μm)	685.5	532.0	833.0	339.0	878.5
Cuticle and epidermal thickness (μm)	5.5	3.5	8.1	3.5	6.7
Diameter of spongy parenchyma (μm)	164.0	113.0	196.0	146.00	166.0
Thickness of scl. plate (μm)	297.5	152.9	286.0	117.5	267.5
Number of scl. layers in scl. plates	8.4	7.5	9.5	6.85	9.25
Thickness of scl. cell wall (μm)	5.4	3.15	8.1	5.45	6.8

*1 = green; 10 = yellow

Table 3 *Correlation coefficients of yellowing and grain yield with various characters in S_1 families of Partap*

Character	Correlation coefficient	
	Yellowing	Grain yield
AGRONOMIC CHARACTERS (100 S_1 FAMILIES)		
Grain yield	—0.265**	—
Stem girth	—0.179	0.084
Plant height	—0.254**	0.652**
Number of leaves	—0.217*	0.610**
Ear-leaf area	—0.197*	0.563**
Sheath length	—0.139	0.053
EPIDERMAL CHARACTERS (100 S_1 FAMILIES)		
Stomatal size	—0.439**	—0.181
Stomatal frequency	0.033	—0.342**
Silica cell size	—0.203*	—0.024
Silica cell (and micro-hair) frequency	—0.059	0.331**
Microhair size	—0.026	—0.022
Macrohair size	0.042	—0.046
Macrohair frequency	0.110	0.316**
ANATOMICAL CHARACTERS (28 S_1 FAMILIES)		
Thickness of lamina	—0.565**	0.032
Thickness of cuticle and epidermis	—0.789**	0.108
Diameter of spongy parenchyma	0.135	0.020
Thickness of scl. plates	0.018	0.002
Number of scl. layers	—0.512**	0.019
Thickness of scl. cell wall	—0.761**	0.036

*, **Significant at 5 and 1 % levels, respectively

temperature is on the differentiation of epidermal cells resulting in a change in stomatal frequency. Thickness of lamina, cuticle and epidermis and sclerenchymatous cell wall and number of sclerenchyma layers were also significantly correlated with yellowing. Cold tolerance was positively correlated with greater expression of these traits.

Palta and Li (1979) reported that in hardy species of *Solanum*, leaves were thicker and smaller. Huner et al. (1981) stated that growth temperature does affect both the leaf thickness and gross anatomy. The thick lamina absorbs a high percentage of incoming radiation because it has higher chlorophyll content per unit leaf area (Friend 1961). Increase in thickness of sclerenchymatous cell wall and number of layers in sclerenchymatous plates may be explained in light of mechanical resistance offered by thickened cell wall in cold tolerant plants (Haberlandt 1965).

Grain yield was positively and significantly correlated with plant height, number of leaves and ear-leaf area, indicating that high yielding S_1 lines were taller, had more leaves and higher ear-leaf area. Plant height had positive and significant correlations with the yield (Sharma et al. 1982). Maize stalk also functions as a storage organ for soluble solids, consisting chiefly of sucrose, that may contribute to grain yield (Hume & Campbell 1972). Increase in number of leaves and ear-leaf area is understandable in terms of increase in assimilate supply to the kernel. Leaves, being the major sites of photosynthetic activity, appear to have an obvious relation to plant's grain yielding ability (Eik & Hanway 1966). In the present case, the positive correlation of plant height, number of leaves and ear-leaf area with yield may partly be due to the fact that a maximal expression of these traits also promoted cold tolerance, and cold tolerant types gave higher yield.

Out of eight epidermal traits, stomatal frequency showed a significant negative correlation with grain yield whereas the frequency of silica cell and macrohair were positively, significantly correlated with grain yield. Kaufman et al. (1979) and Takeoka et al. (1979) suggested that silica cells acted like windows in epidermal system and allowed more light to be transmitted and this was of great significance to total amount of carbon fixed by photosynthesizing mesophyll cells in leaves. Thus, increase in silica cell frequency may be important in terms of increase in total photosynthesis.

In conclusion, there appears to be an appreciable variability in Partap for agronomic, epidermal and anatomical characters. Thus, it should be possible to improve Partap for cold tolerance and grain yield. Both these traits had the desirable correlation. Some epidermal and anatomical traits were significantly correlated with cold tolerance and of these, cuticle and epidermis thickness and stomatal size can easily be measured and used to develop germplasm for cold tolerance.

Acknowledgements

The authors are thankful to Prof. C P Malik, Head, Department of Botany, and to Dr A S Khehra, Head-cum-Senior Maize Breeder, Department of Plant Breeding, for providing the facilities.

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Proc. Indian natn. Sci. Acad. **B51** No. 4 pp 496–504 (1985)

In vitro Culture of *Vanda* Hybrid (*Vanda* TMA × *Vanda* Miss Joaquim). II. Studies on Seedling Explants

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(Received 27 December 1984; after revision 17 July 1985)

Shoot tip meristems below 0.3 mm gave less than 10% response whereas meristems 0.5 mm and above proliferated and produced globular protocorms. Leaves proved highly amenable for tissue culture. Basal region of detached leaves gave multiplication of numerous protocorms in about 50 days. Histological studies revealed that the adaxial surface undergoes differentiation, while the abaxial surface is free from any division. Culture of root segments did not result in callus or organogenesis.

Key Words: *Vanda*, **Leaf regeneration, Protocorm Proliferation**

Introduction

Morel's (1960) attempts to obtain virus free *Cymbidium* plants, using meristem culture have proved a boon to orchid industry. The commercial orchid cultivators utilised this technique for rapid propagation of rare orchid species. Unfortunately, 'mericloning' practised by amateurists often led to the rapid spread of diseased orchid varieties (Langhans et al. 1977). The importance of inoculum size in meristem culture need to be stressed to check this unintentional spread of diseased clones. Leaf tissue culture is feasible for propagation in certain orchids like *Aranda* (Loh et al. 1975), *Cattleya* (Champagnat et al. 1970) and *Paphiopedilum* (Allenberg 1976). Arditti (1977) reviewed the feasibility of clonal propagation in orchids by leaf culture.

Hormonal requirements and culture conditions of the hybrid protocorms of *Vanda* TMA from Bangkok × *Vanda* Miss Joaquim are reported in part I (Mathews & Rao 1985). The *in vitro* responses of the seedling parts of the hybrid are discussed in this paper.

Materials and Methods

Aseptic seedlings of *Vanda* hybrid TMA from Bangkok × *Vanda* Miss Joaquim from Port Moresby Botanical Garden were the source

Abbreviations: *BA*, Benzyl aminopurine; *BTOA*, 2-benzothiazole acetic acid; 2,4-*D*, 2,4-dichlorophenoxy acetic acid; *FAA*, formaldehyde acetic acid alcohol mixture; 2, *i-P*, 6-(r, r-dimethyl allyl aminopurine); *IAA*, indole acetic acid; *IBA*, indole butyric acid; *Kn*, Kinetin; *pCPA*, parachlorophenoxyacetic acid; Z, zeatin

for explants Shoot tip meristems (0·2–0.5 mm long), leaves (5–15 mm in length) root tips and root segments excised from well-developed in vitro raised plants were cultured. The explants were cultured on modified basal medium (Mathews & Rao 1980). Effect of auxins such as IAA, IBA, BTOA, 2,4-D, PCPA, cytokinins BA, Kn, 2, i-P, *Z* and charcoal was tested at different combinations and concentrations in various experiments. *p*H of the medium was adjusted to 5.5–5.6 before solidifying with 0.8% agar (bacteriological grade from Sisco Research Laboratories, Bombay). Plantlets for transplantation were grown on Nitsch's medium (1951) without growth supplements. Cultures were kept under continuous light of 900 lux at 25±2°C with 55–60% humidity. For histological studies, cultured leaves were fixed in FAA. Sections (12 μ thick) were prepared and stained with safranin and fast green.

Results

Shoot Tip Meristem

Meristems of 0.2–0.3 mm length failed to grow on any media whereas explant sizes (0.5 mm) showed a better response.

Effect of Auxins

50% of cultures grown on IAA (1 mg/l) formed green nodular protuberances from the shoot tips. 2, 4-D (1 mg/l) induced yellowsish callus-like proliferation in over 40% cultured shoot tips. Very little proliferation of shoot tip explants was observed on media supplemented with IBA, BTOA and PCPA (table 1).

Effect of Cytokinins

Shoot tip explants cultured on medium supplemented with cytokinins resulted in formation of protocorm units (table 1). In 2,i-P-containing medium, 92% cultures produced green protocorm units. The effect of

Table 1 *Responses of shoot tip (0.3–0.5 mm in size) to various auxins, cytokinins and auxin + cytokinins*

Media	% response	Type of response
Modified basal medium (BM)	0	—
AUXINS		
BM + NAA (1)	0	—
BM + IAA (1)	50	Nodular protuberances Green in colour
BM + 2,4-D (1)	41.67	Yellowish callus like growth
BM + IBA (1)	8.33	Slight proliferation
BM + BTOA (1)	20.83	
BM + PCPA (1)	8.33	
CYTOKININS		
BM + BA (1)	50	Proliferation yellowish in colour
BM + Kn (1)	33.33	Nodular proliferation greenish yellow
BM + 2, i—P (1)	91.67	Green proliferated units
BM + Z (1)	16.67	Yellowish proliferation
AUXIN + CYTOKININ		
BM + IAA (1) + BA (1)	50	Proliferation more callus like
BM + IAA (1) + 2,i-P (1)	83.33	Proliferation—protocorm units
BM + NAA (1) + BA (1)	16.67	Callus growth
BA + NAA (1) + 2,i-P (1)	16.67	Slight proliferation
BM + 2,4-D (2) + Kn (0.5)	0	—
BM + IAA (1) + BA (2)	0	—
BM + IBA (2) + BA (0.5)	0	—

No. of cultures 12; Period of culture 60 days; Concentration of hormones mg/l

various cytokinins on shoot tip proliferation was in the following order Z<Kn<BA<2, i-P.

Effect of Auxin-Cytokinin Interaction

Out of the various auxin and cytokinin combinations, IAA and 2,1-P each at 1 mg/l proved best for shoot tip explants (table 1). The explants proliferated into a number of protocorm units. Shoot tip explants cultured on 2,4-D+Kn, IAA+BA and IBA+BA did not respond and necrosed.

Effect of Charcoal

Charcoal alone at 1% or 2% and with NAA (1 mg/l) did not have any stimulatory effect on the growth of shoot tip. In general, the rate of proliferation in the cultured shoot tip was much slower in comparison to protocorm cultures. After 4 months, the proliferated mass obtained from shoot tip explants was more or less similar to two months old culture of protocorms. None of the media tested induced direct plantlet regeneration from shoot tips.

Culture of Entire Leaves

Random combinations of media tested on culture of entire leaf explants showed that: (*i*) the leaf base was the most amenable region for growth, (*ii*) auxin or cytokinin alone did not stimulate proliferation, (*iii*) auxin and cytokinin combination induced growth and differentiation of leaf, (*iv*) CM alone or in combination with auxin was detrimental, and (*v*) charcoal did not have any stimulatory effect on the growth of leaf explants.

Combinations of IAA+BA, IAA+2,i-P, NAA+BA and NAA+2,i-P (1 mg/l) induced proliferation of protocorms at the base of the leaf explant (figure 1). However, when varying concentrations of 2, i-P and BA in combination with IAA were employed, the cultured leaf explants developed callus preceding the formation of protocorm units. Callussing tedency was more in combinations of IAA+BA (table 2).

The main region of response in leaf was the basal meristem. Young leaves responded better than old leaves. Leaves about 1–2 cm long gave about 90% response compared with the maximum 40% response obtained with bigger leaves. The proliferated basal part formed many protocorm units which could be isolated and grown on multiplication medium and subsequently transferred to Nitsch's medium for obtaining plantlets. If the differentiated units of protocorms were not separated from the leaf base, they continued growth forming leaves and roots while still attached to the original leaf explant (figure 2).

Regeneration of protocorm units from other parts of the leaves was occasionally observed. Figure 3 shows proliferation at base as well

Table 2 *Effect of BA or 2, i-P in combination with IAA on leaf explants*

Treatment	% Response	
BM+IAA(1mg/l)+ Cytokinins	Cultures with more than 10 differentiating units	Cultures showing mainly callus and 2-3 differentiating units
2,i-P (1)	50	50
2,i-P (2)	62.5	37.5
2,i-P (4)	41.67	41.67
2,i-P (8)	45.83	54.17
BA (1)	8.3	91.66
BA (2)	16.6	83.34
BA (4)	4.17	87.5
BA (8)	29.17	70.83

Length of leaf explants, 1.5–2 cm; Growth period, 60 days; No. of replicates, 24; Concentration of growth substances, in mg/l

as tip regions while mid portion was free. Differentiation from the margin is shown in figure 4. Sometimes the proliferation occurred uniformly throughout the length of the leaf explant or was restricted to the mid portion, base and tip being free (figures 5, 6).

Morphogenetic Potential of Isolated Leaf Parts

Leaf base, tip and mid lamina were isolated and cultured on BM + IAA (1 mg/l) + 2,i-P (2 mg/l). Responses are summarised in table 3. Entire leaf and longitudinal half of leaf proliferated at the base and produced protocorms. Over 80% of the isolated leaf base also proliferated. Isolated leaf tip responded rarely. Regeneration of leaf tip was infrequent and was more common when entire leaf was used. Mid lamina portion did not respond at all. Macerated leaf base proliferated into an yellowish tissue with many nodular protuberances (figure 7).

Table 3 *Regenerative potential of isolated leaf parts cultured on BM + IAA (1 mg/l) + 2,i-P (2 mg/l)*

Explants	% response	Type of response
Entire leaf	91.67	Leaf base proliferation and production of protocorm units
Longitudinally split half of leaf	91.67	Proliferation of protocorm units was restricted to the base except in 2 cultures where leaf tip and sides also showed proliferation
Transverse segment of leaf base	83.33	Proliferation and production of protocorm units
Transverse segment of eaf tip	8.33	Occasional production of protocorm units
Transverse segment of mid lamina	0	Explants necrosed
Macerated leaf base	41.67	Slight callus formation

Growth period, 3 months; No. of repicates, 12

Histological Studies

Transverse sections of 10-day-old-culture of leaf indicated meristematic activity on the epidermis, and at subjacent layers of epidermis. Pyramidal structures comprising 6-8 cells arranged in two vertical rows were observed just below the epidermal layer (figures 8,9,10). Sections from 20-day-old leaves showed globular protocorm bodies protruding from the epidermis (figure 11). The protocorm bodies differentiated into leaf and shoot tip (figures 12,13). In 50–60 days old cultures, the whole adaxial surface was crowded with numerous differentiated shoots (figure 14). With prolonged culture, the axil of the primordial leaves showed meristematic activity indicating production of many more shoots. The abaxial leaf surface did not show any tendency for proliferation.

Culture of Root Explants

Root tips (1 mm long) and root segments (3–4 mm long) were cultured on BM + IAA (2 mg/l) + 2, i-P (2 mg/l) and BM + IAA (2 mg/l) + BA (2 mg/l). The explants did not respond and necrosed.

Transplantation of Plantlets

Rooting always accompanied shoot development irrespective of the medium. Plantlets with 4-6 leaves were transferred successfully on to sphagnum moss or to pots filled with charcoal and brick pieces (figure 15). Starting from the seed, it took 15–18 months for obtaining transplantable seedlings. *In vitro* leaf culture gave transplantable seedlings in 10–12 months.

Discussion

Shoot explants were largely used in earlier studies of orchid tissue culture (Rao

1977, Goh 1983). In the present study on *Vanda*, meristem explants below 0.3 mm in length did not respond. The responses of shoot tip explants to growth supplements showed that cytokinins alone as well as auxin+cytokinins favoured growth. Auxin alone did not enhance the growth of meristems. It is possible that the meristems are rich in endogenous auxins and hence cytokinins in the medium stimulated growth while exogenous auxins proved supraoptimal. Combinations of auxin and cytokinin favouring growth of shoot tip meristem have been reported in *Cattleya* (Kusumoto 1979a). The rate of proliferation in shoot tip explants was much slow compared to the protocorm or leaf cultures. Hence mericloning should be resorted to only when virus-free plants are desired.

The first report of orchid leaf proliferation was of the embryonic leaves of *Cymbidium*. In recent years, leaf cultures have been attempted in many orchid species such as *Aranda* (Loh et al. 1975), *Epidendrum*, *Laeliocattleya* (Churchill et al. 1971a, b), *Paphiopedilum* (Allenberg 1976) *Phalaenopsis* and *Vanda* (Tanaka et al. 1974). Excised leaves from young seedlings were used for vegetative propagation in *Cattleya* (Champagnat et al. 1970). Detached leaves with their intact bases produced shoots and roots in *Echeveria elegans* (Raju & Mann 1971). In the present study regeneration from basal region was more frequent than from leaf tip or margin. Moreover, the leaf tip differentiation frequency was significantly better when entire leaves were cultured rather than in isolated leaf explants, thereby showing that the presence of the leaf base was a deciding factor even for the distal end proliferation. According to Raju and Mann (1971) the exclusive production of roots and the loss of the ability to form shoots when the proximal ends of leaves were removed, indicates the significance of the leaf base. The leaf base in monocots is generally meristematic and on isolation and culture they differentiate plantlets (Zimmer & Pieper 1975).

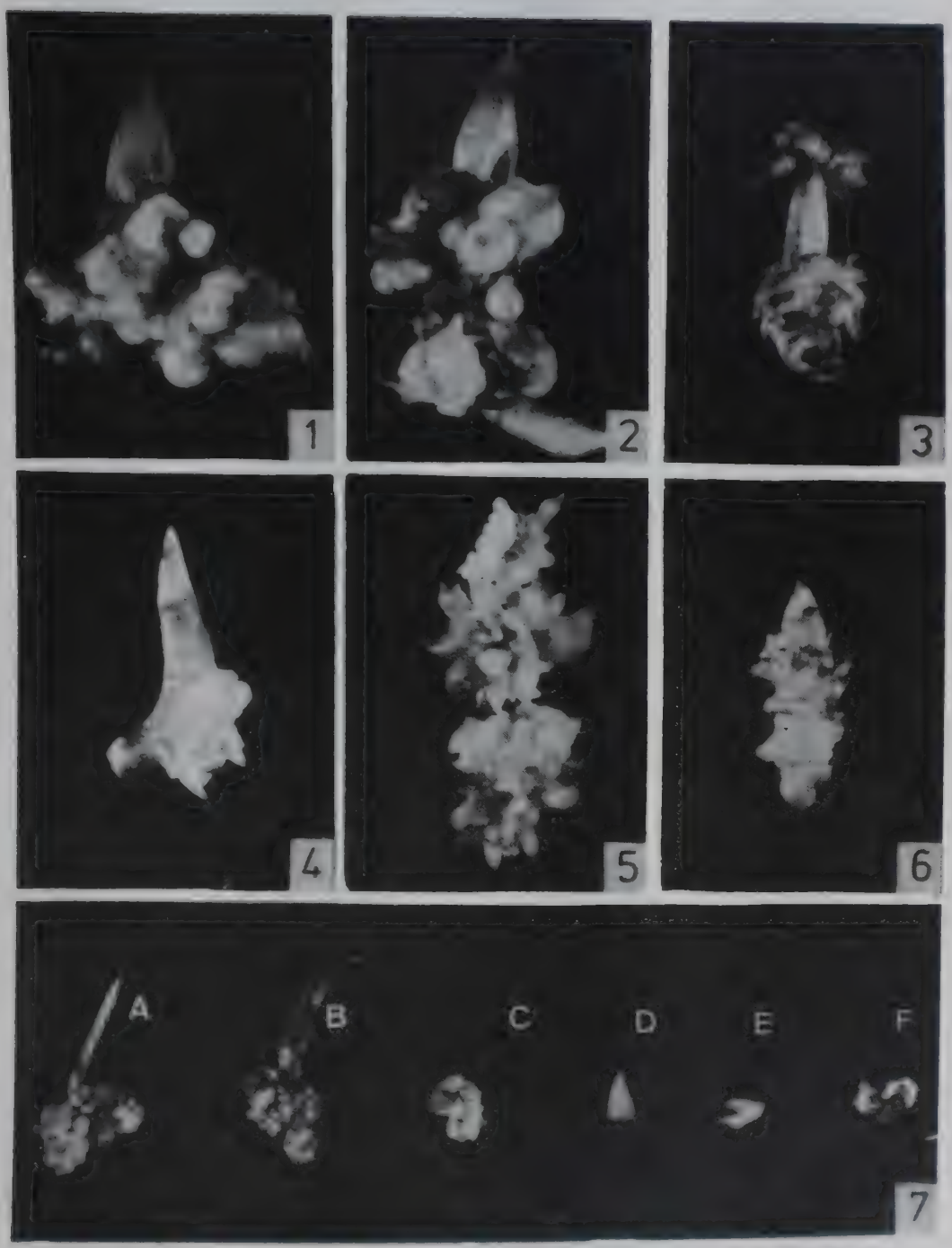
Microtome sections of cultured leaves of *Vanda* showed that the adaxial surface underwent regeneration whereas the abaxial surface did not produce any shoot or protocorm. In *Heloniopsis orientalis*, Kato (1976) reported that bud formation was confined to the ventral surface. In *Begonia* and *Lilium* Bigot (1974) reported that only epidermal cells participate in bud formation, suggesting the existence of predetermined regenerative cells. Meristematic activity was observed by us in both epidermal and sub-epidermal regions, and it is likely that both protocorm bodies as well as direct differentiation of shoot occurred.

Leaf tips of *Epidendrum* and *Laoliacattleya* cultured *in vitro* (Churchill et al. 1973) yielded callus and plantlets. In present studies on *Vanda*, leaf tip regeneration was infrequent. Occasional formation of plants from leaf is reported to be an inherent trait of orchidaceae which is 'turned on' under *in vitro* culture conditions. Once perfected, propagation through leaf culture woud be an ideal method, as removal of small sections does not damage the mother plant and thousands of plants can be produced from a single leaf segment.

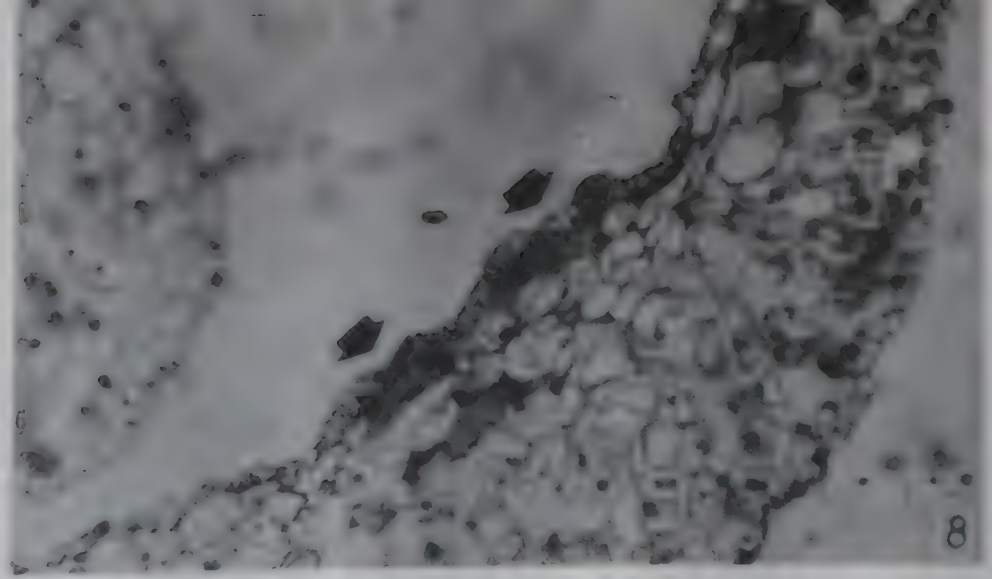
Root tip cultures in the present investigations did not result in callus formation or differentiation. In root cultures of *Phalaenopsis*, Zimmer and Peiper (1978) reported formation of protocorm bodies although the frequency was low. Kusumoto (1979b), using relatively high concentrations of growth regulators, induced the formation of callus like protocorms in *Cattleya*. In *Spathoglottis* (Bapat & Narayanaswamy 1977), rhizoidal callus was obtained on MS medium supplemented with NAA and CM.

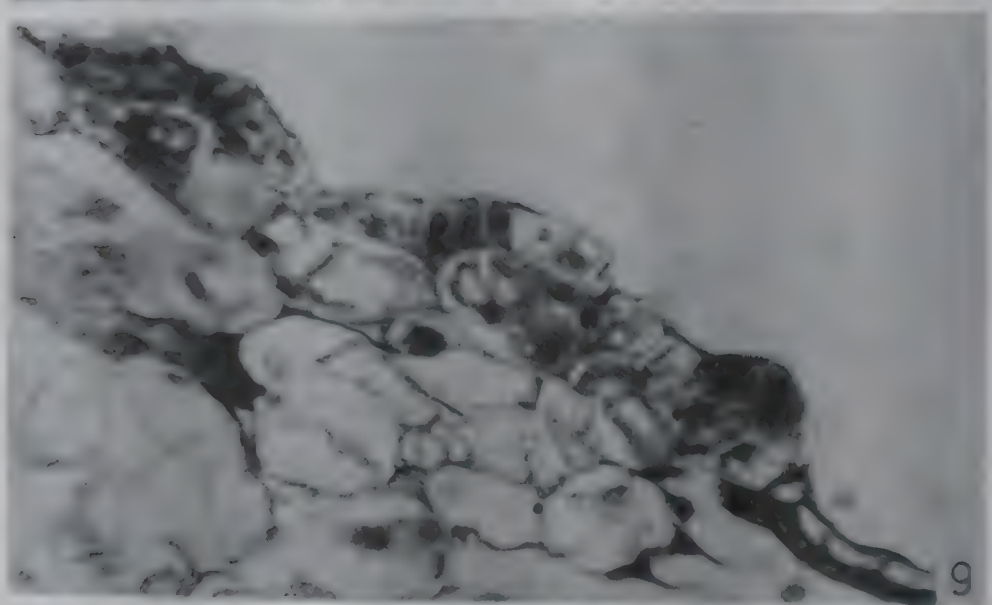
Acknowledgements

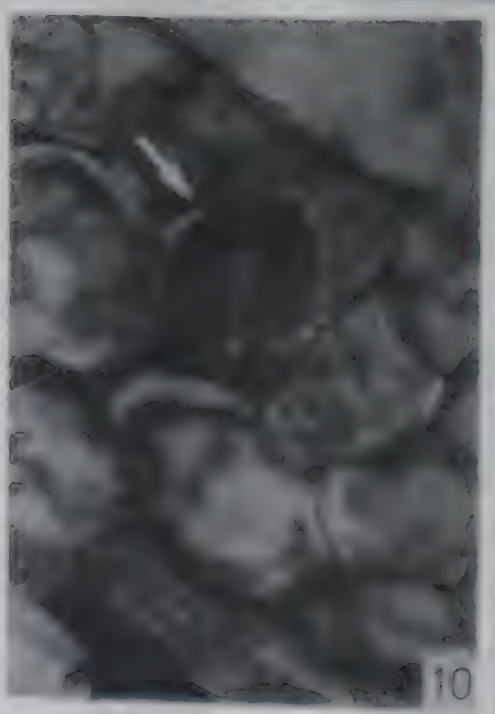
The authors thank Professor A Abraham (Trivandrum) for the supply of pods of *Vanda* hybrid.

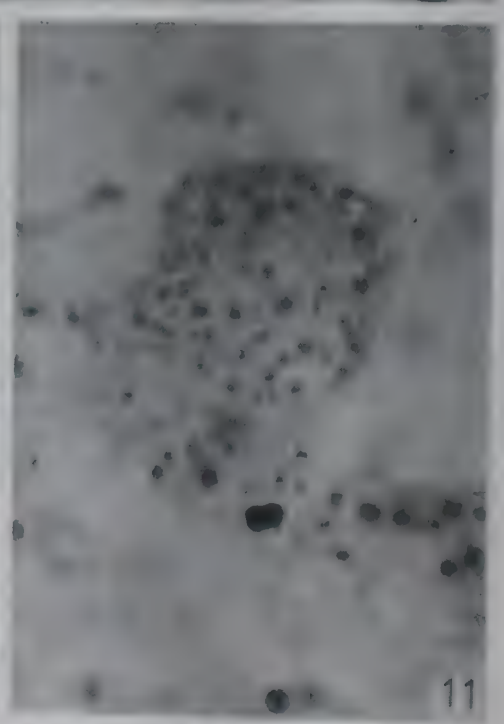


Figures 1–7 Morphogenetic responses of cultured leaves of *Vanda* hybrid. **1,** Differentiation of protocorms from the leaf base; **2.** Development of differentiated units into plantlets while still attached to the leaf explant; **3,4,** Differentiation of protocorms from the base and tip of the leaf explant and from the margin of leaves (Fig. 4); **5,** Protocorm proliferation throughout the length of the leaf explant; **6,** Mid lamina showing proliferation while leaf base and leaf tip are free; **7,** Responses of leaf parts : whole leaf (A, longitudinally split half of leaf (B), leaf base (C), Leaf tip (D), midlamina (E), and macerated leaf base (F)

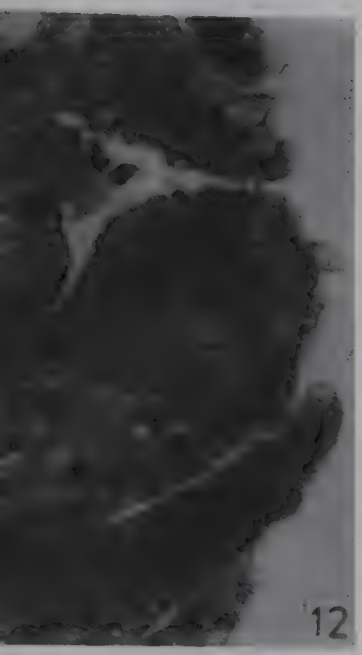








Figures 8–11 Histological studies on the origin of protocorms in cultured leaf explants. **8**, Divisions in the epidermel cells on the adaxial surface of leaf, 8-10 days after culture (shown by arrows); **9**, An enlarged view of a portion from above; **10**, Pyramidal structures with rows of cells, emerging from below the epidermis (see the arrow); **11**, Protocorm body formed in leaf after 15–20 days of cultures



Figures 12–15, 12, Differentiation of shoot tip and primordial leaves; **13,** Differentiation on the protocorm bodies formed in leaf culture; **14,** Numerous shoot regenerants on adaxial surface of leaf about 55–60 days after culture. Note the meristematic regions (darkly stained at axils of the primordial leaves, the abaxial surface is free of any cell divisions); **15,** Hybrid plants of *Vanda* TMA × *Vanda* Miss. Joaquim, multiplied through leaf culture technique growing on sphagnum moss in Trombay nursery

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Proc. Indian natn. Sci. Acad. B51 No. 4 pp 505-510 (1985)

Nuclear DNA and Analysis of Heterochromatin in Different Species and Populations of *Rauwolfia* L.

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(Received 8 March 1985)

Measurement of nuclear DNA contents in *Rauwolfia serpentina* Benth. and *R. canescens* L. revealed significant differences at both inter and intra-specific levels, the variations being more marked in the former than in the latter.

The DNA content of *R. canescens* 2n=66 was nearly double that of *R. serpentina* (2n=22).

The amount of heterochromatin as measured from the interphase blocks in the different populations of the species, was found to bear a more or less constant relation to their nuclear DNA contents. The elimination of heterochromatic segments in the polyploid species does not necessarily involve the centromeric heterochromatin which only was measured.

Key Words: Nuclear DNA, Heterochromatin, *Rauwolfia serpentina*

Introduction

Variations in DNA content in plants both at inter and intraspecific levels are well documented (Bennett 1970, Bullen & Rees 1972, Chooi 1971, Furuta et al. 1975, Jones & Rees 1968, Kadir 1974, Mukhopadhyay & Sharma 1984, Narayan & Rees 1976, Price et al. 1980, 1981, Sharma 1983, Sharma & Chattopadhyay 1983, Sharma & Mukhopadhyay 1984).

The vast difference in DNA contents often noted among related species has been attributed to the supplementary DNA (Ayonoadu 1974, Bennett et al. 1977, Rees et al. 1979, Resslar et al. 1981). According to these authors, this DNA contains mainly amplified non-coding sequences, but also to some extent coding sequences arising through mutational events. This supplementary DNA has a specific organization with a distinct range of tolerance.

Notwithstanding the differences in DNA content noted in different species and

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populations, precise information about the nature of sequences involved in such variations is lacking. Since heterochromatin may have a role in conferring adaptability to a species, a correlation of the total amount of heterochromatin with the total amount of DNA may lead to an insight into the functioning of the former.

The present investigation was undertaken with a view to analyse the DNA content, both at intra- and inter-specific levels and to determine the relationship with heterochromatin. With regard to the latter, only those heterochromatic segments which appear as dark spots at interphase were taken into consideration. The heterochromatic segments which do not show allocycly and/or do not appear as brightly stained bodies during the interphase could not be included within the scope of the present investigation due to their obvious limitation. It was deemed also worthwhile to measure the size and volume of chromosomes in so far as these are related to the total heterochromatic DNA content.

Materials and Methods

The present investigation was carried out on 18 wild and cultivated populations of *Rauwolfia serpentina* Benth., *R. canescens* L. and *R. vomitoria* Afz. collected from different parts of India.

Estimation of nuclear DNA and heterochromatin as interphase blocks

For cytophotometric estimation of nuclear DNA, freshly collected root tips were fixed in glacial acetic acid:ethanol (1:3) for 3 hr. They were then hydrolyzed in (1N) HCl solution at 60°C for 12 to 14 min, thoroughly washed in distilled water, stained in Schiff's reagent for 1 hr and finally squashed in 45% acetic acid. A Reichert Zetopan microspectrophotometer was used and the single wavelength method (Sharma & Sharma 1980) was adopted. Nuclear DNA values were calculated on the basis of optical density in terms of arbitrary units of relative absorbances. For each population 50 observations were recorded from somatic metaphase plates.

To measure the amount of heterochromatic DNA as interphase blocks, feulgen-stained preparations made in a similar way were used. In the interphase nuclei, the constitutive heterochromatin appears as dot-like particles, the number of which varied from 9 to 16 per nucleus in different populations and species of *Rauwolfia*. Optical measurements of individual dots were made for each population and the average absorbance value of an individual dot multiplied by the mean number of dots gave the total absorbance value of heterochromatic DNA measured per interphase nucleus. Finally, the percentage of heterochromatic DNA as interphase blocks was calculated against total nuclear DNA.

Determination of chromosome volume and size

Chromosome volume was calculated assuming each chromosome to be a cylindrical structure using the formula : volume $\pi r^2 \times 1$, where 'r' denoted mean radius of chromosome and 'l' denoted total length of chromosome. The total chromosome length at metaphase of each cell was measured and a mean estimate of chromosome width was obtained from a random measurement of ten chromosomes per cell. All measurements were made from stained preparations under oil immersion objective.

The average chromosome size was determined by dividing the total chromosome length per cell by the total chromosome number per cell.

Results and Discussion

Polyploidy, chromosome size and the amount of nuclear DNA

Altogether 12 populations of *R. serpentina*.

5 of *R. canescens* and 1 of *R. vomitoria* were studied. Except *R. canescens* populations which are hexaploid with 2n=66 chromosomes, all the other populations studied by us were diploid with 2n=22 chromosomes (Banerjee 1982).

Inspite of the uniformity in chromosome number in the different populations of *R. serpentina*, the amount of nuclear DNA showed a considerable degree of variation, ranging between 1.1685 (population II) and 2.0440 units (population I) (table I). The mean value was 1.6082, but one-third of the populations fell within the range 1.31 and 1.50 units and another one-fourth of the populations in the range 1.91 and 2.10 units, thus showing a bimodal distribution pattern. If this distribution pattern is real, it indicated a genetic heterogeneity of the populations used for this study. The variation of nuclear DNA content within a single species is an index of genetic variability and it plays a significant role in diversification. Such variation at intraspecific level has already been reported for other species (Furuta et al. 1975, Miksche 1971, Price et al. 1980).

The amount of nuclear DNA in the 5 populations of *R. canescens* was found to range between 2.3754 in population XV to 3.0958 in the population XVIII (table 2). In view of the high chromosome number (2n=66), this variation is not high. In fact, it is smaller than that in *R. serpentina*. The mean content of nuclear DNA in *R. canescens* is 2.7714 in terms of arbitrary units which is only 1.7 times that of the mean value found in the populations of *R. serpentina*.

R. vomitoria (2n=22) of which only a single population was dealt with, showed similarity with *R. serpentina* in nuclear DNA content and chromosome size and volume (table 1). Variation of DNA content of

Table 1 *Variations in the ranges of chromosome size, chromosome volume, nuclear DNA and heterochromatin content among the populations of* Rauwolfia serpentina *Benth*

Populations 2n=22	Average chromosome size (μm)	Chromosome volume (cu. μm)	Total nuclear DNA (arbitrary units) Mean±SE	Heterochromatin* (arbitrary units) Mean±SE	% heterochromatin in total nuclear DNA
I	2.54	49.28	2.0440±0.0123	0.6024±0.0181	29.47
II	2.28	42.68	1.1685±0.0154	0.6249±0.0296	53.47
III	2.93	66.43	1.8852±0.0105	0.7790±0.1322	41.32
IV	2.66	54.99	1.6769±0.0007	0.5619±0.0224	33.50
V	2.76	64.44	1.5042±0.0102	0.5681±0.0182	37.76
VI	2.99	63.90	2.0162±0.0095	0.6007±0.0236	29.79
VII	2.57	48.06	1.3812±0.0075	0.5917±0.0236	39.81
VIII	2.79	69.49	1.4026±0.0089	0.5096±0.0141	36.33
IX	3.39	60.51	1.2856±0.0077	0.5653±0.0065	43.97
X	2.52	69.50	1.3177±0.0086	0.6460±0.0122	49.02
XI	2.98	63.63	1.6698±0.0081	0.6460±0.0122	38.77
XII	2.87	63.12	1.9482±0.0098	0.8538±0.0347	43.82

*Heterochromatin measured as interphase blocks.

Table 2 *Variations in the ranges of chromosome size, chromosome volume, nuclear DNA and heterochromatin content among the populations of* Rauwolfia canescens *L. and* R. vomitoria *Afz.*

Populations of *R. canescens* 2n=66	Average chromosome size (μm)	Chromosome volume (cu./μm)	Total nuclear DNA (arbitrary unit) Mean ± SE	Heterochromatin* (arbitrary unit) Mean ± SE	% heterochromatin in total nuclear DNA
XIV	1.79	78.25	2.5957±0.0052	1.2764±0.0346	49.17
XV	1.54	76.79	2.9350±0.0086	1.2718±0.0332	43.33
XVI	1.67	70.45	2.3754±0.0044	1.1726±0.0655	49.36
XVII	1.61	63.45	3.0958±0.0082	1.1818±0.0223	38.17
XVIII	1.60	58.51	2.8338±0.0074	1.3793±0.0806	48.67
R. vomitoria (2n=22) Population XIII	2.57	54.99	1.4248±0.0071	—	—

*Heterochromatin measured as interphase blocks

different species of a genus has been studied by many others (Ayonoadu 1974, Bennett et al. 1977, Bullen & Rees 1972, Chooi 1971, Gupta 1976, Jones & Brown 1976, Jones & Rees 1968, Kadir 1974, Narayan & Rees 1976, Resslar et al. 1981, Sharma & Mukhopadhyay 1984).

So far as chromosome size is concerned, *R. canescens* populations show an overall reduction in comparison to the populations of *R. serpentina* and *R. vomitoria* (table 1 & 2). Whereas, the average chromosome size ranges from 1.60 to 1.79 μm in the *canescens* populations, it is between 2.28 to 3.39 μm in the populations of the other two species. Reduction in chromosome size in polyploids is, however, not uncommon in plant systems (Sharma & Sharma 1959, Sharma 1972), though the mechanism through which reduction is achieved is not well known. Various factors, like heavy condensation and inactivation of gene segments in polyploids, loss of heterochromatic segments, or differential polynemy have been supposed to be the causes.

From the data, it is difficult to state how far polynemy is responsible for reduction of chromosome size in the polyploid species (*R. canescens*), but a comparison of the values of mean radius and chromosome volume of the diploids and the hexaploid at least does not indicate any such behaviour. Condensation of the chromosome thread and deletion of heterochromatic segments might have played a role in the overall reduction of chromosome size in the polyploid species of *Rauwolfia*.

Contents of DNA and heterochromatin in species of Rauwolfia

It is remarkable that in *R. canescens* in which the chromosome number is three times that of *R. serpentina* or *R. vomitoria*, the total amount of DNA is not three-fold (tables 1 & 2). The ratio varies rather widely between 1.16 to 2.60 in different populations of these species. The absence of a proportionate increase in DNA content commensurate with the chromosome number in the hexaploid species may be due to the elimination of heterochromatic segments. It has

been reported for *Allium stracheyii*, where heterochromatic segments become completely eliminated in polyploids (Sharma & Aiyanger 1961). It has been assumed that the adaptive advantage conferred by the heterochromatic regions becomes redundant in the polyploids where the same function is performed by the increased gene dosage. However, *R. canescens* has probably a hybrid origin, as indicated by the absence of multivalent formation (Banerjee 1982).

To probe into the cause of a lower content of DNA than the expected level in the hexaploid species, i.e. whether it can be explained by the loss of heterochromatic segments, the amounts of DNA in heterochromatin appearing as heterochromatic blocks in the interphase nuclei in the diploids and hexaploids were compared (tables 1 & 2). It can be observed from the data that the amounts of heterochromatic DNA do not show any marked difference. In fact, the percentage of heterochromatic DNA in total DNA shows a much wider variation within the populations of *R. serpentina* (29.47% to 53.47%) than between *R. serpentina* and *R. canescens*. It should be remembered, however, that absence of a difference in heterochromatin measured as interphase blocks does not totally preclude the possibility of elimination of heterochromatic segments in the polyploids. Because, the allocyclic behaviour in the interphase mostly involves the centromeric heterochromatin, whereas the major intercalary portions may not follow suit. With the cytophotometric method used in the present study, it was not possible to detect heterochromatin which did not form interphase blocks. Chromosome banding technique and analysis of repetitive DNA sequences might provide more useful information about such elimination. From the present investigation it can be concluded that the centromeric heterochromatin remains virtually the same in quantity in both the diploid and hexaploid species of *Rauwolfia*.

From the chromosomal behaviour in meiosis it has been assumed that the hexaploid species might have originated by hybridization, though it must be admitted that no diploid type of *R. canescens* has so far been discovered. If the progenitor had a considerably lower DNA content than *R. serpentina*, the values of *R. canescens* would not then appear so disproportionate. This is on the basis of the assumption that the ancestral type had the total chromatin length and the total DNA comparable to that of *R. serpentina*.

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Proc. Indian natn. Sci. Acad. **B51** No. 4 pp 511–514 (1985)

Oil Content and Fatty Acid Composition of Mustard Seed (*Brassica juncea* L) obtained through Tissue Culture

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Oil content and fatty acid composition of seed oil of *Brassica juncea* cv. Rai-5 obtained *in vitro* on cotyledon explants from non-irradiated and irradiated seeds were determined after growing the plants in the field for two generations. Oil content was determined by pulsed NMR technique. An increase in oil content was observed in the yellow variants obtained from cotyledons of non-irradiated seeds. Fatty acid composition of the seed oil from various plants was determined by GLC. Although some reduction was noticed in the erucic acid content of some plants, most of them had the fatty acid composition comparable to that of the donor material Rai-5.

Key Words: *Brassica juncea*, **Fatty acid analysis, Oil content, Tissue culture**

Introduction

Indian mustard (*Brassica juncea* L) is an important oil seed crop grown in many countries as a source of vegetable oil. The seed oil of mustard as well as that of rape (*B. napus*) and turnip rape (*B. campestris*) contain very high levels of erucic acid which make them less desirable as a dietary fat. Demand for improved oil quality has led to the development of zero erucic acid varieties of rape which in many countries (Jonsson 1977) have replaced the erucic acid containing types. Mustard is a very popular oil crop in India and almost all the cultivars under cultivation have high erucic acid levels (Anand 1975). Tissue culture of *Brassica juncea* var. Rai-5 was initiated in this laboratory with the objective of isolating genetic variants with low erucic acid content. The present communication briefly describes the results obtained so far.

Materials and Methods

Earlier we (George & Rao 1980) have reported the *in vitro* regeneration of mustard plants (*Brassica juncea* var. Rai-5) on cotyledon explants from non-irradiated, irradiated and mutagen-treated seeds. The seeds were aseptically

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germinated on sucrose-agar medium and cotyledons excised from 8-day old seedlings were cultured on Murashige and Skoog's basal medium (MS, Murashige & Skoog 1962) containing naphthalene acetic acid (NAA 1 mg/l) and 6-benzylaminopurine (BA 1 mg/l). The regenerated shoot buds were isolated and rooted on MS basal medium containing NAA (1mg/l). Complete plants thus obtained were first transferred to vermiculite in paper cups and later planted in soil.

The plants were harvested individually and the seeds were collected. During the next season, all the plants were grown in the field in isolation and the yellow variants were selfed to avoid cross pollination. Seeds were collected separately from each plant for further studies (R_2 generation).

The yellow variants were grown for five more generations and were found to be true breeding.

Estimation of Seed Weight

Hundred seed weight was determined by weighing 100 seeds in four replicates selected at random from the bulked seeds of each line. This was then computed to 1000 seed weight and the mean was calculated.

Estimation of Oil Content

The oil content of the seeds was determined by pulsed Nuclear Magnetic Resonance (NMR) technique (Tiwari et al. 1974, Srinivasan 1979). In this method, the NMR signal intensities of the different types were compared with that from a known amount of chemically extracted oil from Rai-5 seeds and oil percentages have been calculated on basis of wet weight.

Fatty acid Analysis

One hundred mg of mustard seeds were crushed in a small mortar with 2 ml of petroleum ether. The organic layer was filtered through a mimicolumn of anhydrous sodium sulphate into a 10 ml volumetric flask. This process was repeated four times. Total volume was made up to 10 ml.

The oil (obtained by evaporation of 2 ml of petroleum ether extract) was mixed with 0.1 ml of dry benzene, 0.2 ml of dry methanol and 0.5 ml of boron trifluoride-methanol. It was kept at 80–90°C for about 30–40 min. The solvents were evaporated off. The residue was taken in petroleum ether and washed with water. The organic layer was passed through a minicolumn containing anhydrous sodium sulphate. The weight of the methyl esters formed was noted.

Fatty acid analysis of the seed oil was carried out on Toshniwal Gas liquid Chromatograph using glass column (6×6mm OD) containing 10% Silar 7 C on gas chrom Q (80/100). The corresponding temperatures were column; 185°C, injection: 240°C and detector: 240°C.

Results and Discussion

Although about 200 plants obtained from cultured cotyledons were planted in soil, all of them did not survive. About 66% of the plants from non-irradiated and 50% from irradiated seeds survived and grew in the field, eventually producing flowers and seeds (R_1 generation). Plants obtained from the mutagen-treated seeds (seeds treated with ethyl methane sulphonate, EMS) did not survive. All the plants were grown in isolation to prevent cross pollination.

Morphologically, the plants obtained *in vitro* were similar to Rai-5 having blackish-brown seeds and being true breeding served as the source material for culture. However, among the 85 plants raised from untreated seeds (from cotyledons of non-irradiated seeds), 7 had yellow seeds and the remaining ones had blackish-brown seeds. All the plants raised from cotyledons of irradiated seeds had blackish-brown seeds.

Seed Weight

Thousand seed weight was calculated from each line to study if tissue culture and/or irradiation had any effect on this trait and also to ascertain if there existed any correlation between seed weight and oil content. Wide variation was observed in the seed weight of the lines (blackish-brown type) derived from non-irradiated seeds (table 1). It varied from 1.9 g to 2.8 g per 1000 seeds, average being 2.0 g. The yellow variants had a lower and more uniform seeds weight (1.3 to 1.7 g per 1000 seeds) with a mean value of 1.6 g. The average seed weight was 2.1 g in the irradiated types and there was not much variation among the various lines (1.8 to 2.3 g per 1000 seeds).

Oil Content

The oil content of the seeds obtained from R_2 progeny was determined by pulsed NMR technique. Rai-5 (the standard cultivar) had 26.8% oil in the seed (table 1). Plants obtained from cotyledons of non-irradiated seeds were divided into two groups: the blackish-brown types and the yellow variants. The oil content in the blackish-brown types was 23.9% whereas in the yellow variants it was 29%. The oil content in plants raised from cotyledons of irradiated seeds was about 22.6%.

In general, plants raised through tissue culture (blackish-brown type and irradiated type) had lesser oil content compared to the standard cultivar. There was no correlation between seed weight and oil content in these groups. However, the yellow variants had a lower seed weight and higher oil content. In *Brassica* lower seed weight within a certain range is associated with higher oil content (Olsson 1960). Similarly in mustard as well as in rape the yellow seeds have thinner seed coats and hence a higher oil content than blackish-brown types (Kirk & Oram 1978, Jonsson 1977).

Fatty acid Composition

In seed oil from plants raised from cotyledons of non-irradiated seeds : blackish-brown type

The fatty acid composition of oil from Rai-5 and blackish-brown types, obtained from untreated seeds through tissue culture, is presented in table 1. The major fatty acids in the standard cultivar Rai-5 and *in vitro* obtained plants were oleic, linoleic, linolenic, eicosenoic and erucic acids and these together contributed to about 94–96% of the total fatty acids. Among the major fatty acids, erucic acid alone contributed to 45.1% in Rai-5. Concentration of this acid varied from 37.5% to 46.4% in the *in vitro* obtained plants and the average value was 40.5%.

In seed oil from yellow variants obtained through tissue culture

No major differences were observed between the blackish-brown types and yellow variants with regard to the concentration of various fatty acids. Erucic acid was about 41.4% in the yellow variants.

In seed oil from plants obtained from cotyledons of irradiated seeds

All the 16 plants raised from cotyledons of irradiated seeds produced blackish-brown seeds. Oil analysis was carried out with

Table 1 *Seed weight*, oil content* and erucic acid concentration* of mustard seeds* (Brassica juncea *L. var. Rai-5*) *obtained through tissue culture from cotyledons of non-irradiated and irradiated seeds — R_2 generation*

Line	Wt/1000 seeds gm	Oil content per cent	Erucic acid per cent
Rai-5 (control)	1.70	26.80	45.10
Coty. non-irr. (12 lines)	1.99–0.08*	22.89–0.51*	40.47–0.63*
Yellow variants (6 lines)	1.55–0.07	28.95–0.68*	41.44–1.38*
Irradiated (10 lines)	2.70–0.07*	22.74–1.38*	41.52–1.31*

*The values are significantly different from the corresponding values of Rai 5 (control) (level of significance–5%)

10 types. The average level of erucic acid was about 41.4% (table 1).

The results obtained from the present study suggest that in *B. juncea* it is possible to obtain stable mutants such as yellow variants through tissue culture technique. These results also indicate that in this species somatic tissue culture may not be the ideal method for the isolation of zero erucic acid lines although a few plants had a lesser concentration of this acid than the donor material. Experiments are in progress to study the agronomic characters of the *in vitro* obtained plants and to ascertain whether they are superior to the existing varieties with regard to yield and other characters.

Acknowledgements

The authors thank Dr M S Chadha and Dr P S Rao, Bio-Organic Division, for their kind encouragement during this work.

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Proc. Indian natn. Sci. Acad. B51 No. 4 pp 515-521 (1985)

Stepwise Hydration of Soybean Seed to Prevent Soaking Injury and Loss of Viability during Storage

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(Received 23 February 1985; after revision 26 August 1985)

Soaking of soybean (*Glycine max* cv. Bragg) seed for a brief period followed by drying back failed to improve its germinability due to rapid imbibition and consequent damage. Stepwise hydration of the seed was, therefore, attempted to reduce membrane damage and leakage loss during hydration. Osmotic control by two-step hydration (osmotic treatment with PEG — soaking in water) followed by drying showed improvement in immediate germination, whereas three-step hydration (moisture equilibration — osmotic treatment with PEG — soaking in water) followed by drying showed remarkable effect on storability; soaking-drying treatment of humidified seeds also showed promising effect.

Key Words: Osmoconditioning, Moisture equilibration, Polyethyleneglycol, Vigour, Conductivity of seed leachate, Membrane damage

Introduction

Germination requires that seeds be hydrated. Hydration may occur as absorption from liquid water or it may, in certain instances, suffice for the seeds to be placed in a humid atmosphere, where they will gradually establish a level of hydration suitable for germination. Seed soaking, a practice often resorted to by seed growers for hastening germination and making it more uniform (Kay et al. 1977), does not always bring about the desired results. In fact, in legume seeds, (particularly in soybean), soaking directly in water is seldom beneficial (Barton 1950a, b, Orphanos & Heydecker 1968) as rapid imbibition can injure seeds (Powell & Matthews 1978, 1979). Barton (1959a, b) observed that high concentration of oxygen may cause severe soaking injury. In such cases, regulating the process of hydration *per se* by osmotica has been attempted by several workers with varied degrees of success (Khan 1977, Heeperly & Sinclair 1977, Khan et al. 1978). Soaking-drying treatment of stored seed is reported to maintain viability in several cereals, oilseeds and vegetable seeds in storage (Basu 1976). It is now becoming apparent that hydration of a dry seed preparatory to germination is after all not a simple and straightforward phenomenon and that physico-chemical

forces thus initiated in the material and structures of the seed have a role far more significant than hitherto appreciated. This paper presents several methods of preconditioning soybean seeds with a view to overcoming possible soaking injury during treatment and thus prolonging storage life and vigour.

Materials and Methods

Soybean seeds (*Glycine max* L. cv. Bragg) harvested in March, 1982 were cleaned and dried to 9.0% moisture (w.wt.). They were stored in sealed glass bottles (2.5 litres) at room temperature (av. 30±3°C) till the time of treatment.

Seed Treatment

The soaking-drying treatment was carried out according to the following methods. The technique is shown in figure 1.

A. Water soaking-drying (WS-D). Seeds were soaked in distilled water double their volume for 3 hr at 28°C, and then dried back to their original moisture content.

B. Polyethylene glycol-water soaking-drying (PEG-WS-D). Seeds were soaked in different concentrations (2–30%) of polyethylene glycol (mol. wt. ca 6000) solutions double their volume at 28°C for 2 hr followed by soaking for an additional hour in distilled water, and then dried back to their original moisture content.

C. Moisture equilibration-water soaking-drying (ME-WS-D). Seeds were subjected to moisture equilibration for 48 hr in a saturated atmosphere (100% RH) at 28°C following the method of Dey and Basu (1982). These were then treated as in A; that is, the full treatment was moisture equilibration-water soaking-drying.

D. Moisture equilibration-polyethylene glycol-water soaking-drying (ME-PEG-WS-D). Seeds were treated as in B except that before soaking in PEG and distilled water, seeds were equilibrated in a saturated atmosphere at 28°C for 48 hr, that is, the full treatment was moisture equilibration-polyethylene glycol-water soaking-drying.

Before drying, one part of each treated lot was placed for germination following the glass plate method of Punjabi and Basu (1982). The other part was dried in a current of hot air at 35 ± 1°C to its original moisture content and stored. Determination of water absorbed during different stages of soaking was made following the method of Knypl and Khan (1981). Initial moisture content of the seed and moisture level attained after equilibration in a saturated atmosphere were determined by oven-drying method.

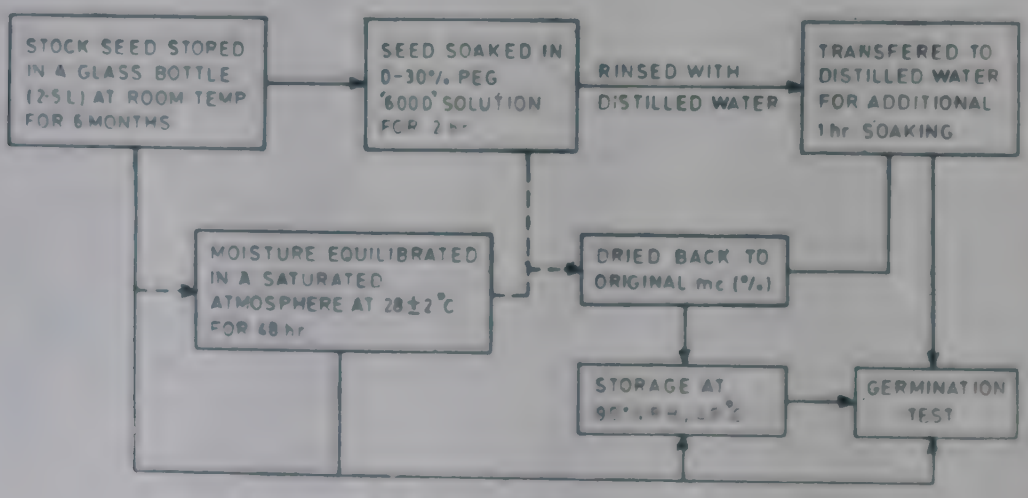
STOCK SEED STORED IN A GLASS BOTTLE (2·5 L) AT ROOM TEMP FOR 6 MONTHS
SEED SOAKED IN 0-30% PEG '6000' SOLUTION FOR 2 hr
RINSED WITH DISTILLED WATER
TRANSFERED TO DISTILLED WATER FOR ADDITIONAL 1 hr SOAKING
MOISTURE EQUILIBRATED IN A SATURATED ATMOSPHERE AT 28±2°C FOR 48 hr
DRIED BACK TO ORIGINAL mc (%)
STORAGE AT
GERMINATION TEST

Figure 1 Methods of osmotic treatment of soybean seed

Leakage of metabolites at different steps of hydration of equilibrated and nonequilibrated seeds was tested following the method of Dey and Basu (1982).

Results

Germination and seedling growth were found to be better in moisture equilibrated seeds as compared to dry seeds directly placed for germination (figure 2). Water soaking failed to show any detectable improvement in germination over dry placement or moisture equilibration of seeds. Pretreatment of seeds with PEG '6000' (2–30%) for 2 hr followed by 1 hr soaking in distilled water improved percentage germination over pretreatment with water alone (0% PEG). However, only at concentration above 20% PEG, germination was greater than after ME alone. Polyethyleneglycol at 22.5 and 30% showed the maximum beneficial effect.

Seed vigour (seedling growth) was improved by ME alone over dry control or 3 hr water soaked control (figure 2). Treatment with PEG improved shoot length up to 22.5% PEG beyond which the effect declined. Little difference in root length was noticed between moisture equilibrated seeds and seeds treated with the higher concentrations of PEG (22.5 or 30%).

When moisture equilibrated seeds (m.c. 21.0%, w.wt.) were soaked for 3 hr in water or in different concentrations of polyethyleneglycol solutions for 2 hr followed by an additional 1 hr soaking in water, an enhancement of germination was noticed in all concentrations of PEG over only moisture equilibrated or untreated dry controls. Although the treatments were better than PEG alone, the effects were found to depend on the concentration of PEG, maximum improvement being noted at 2 and 5% with the effect declining at higher PEG concentrations. The vigour of the seedlings was better in ME followed by PEG than in ME followed by water treatment. The improvement in root length followed the pattern of germination and declined at and beyond 10% PEG; shoot

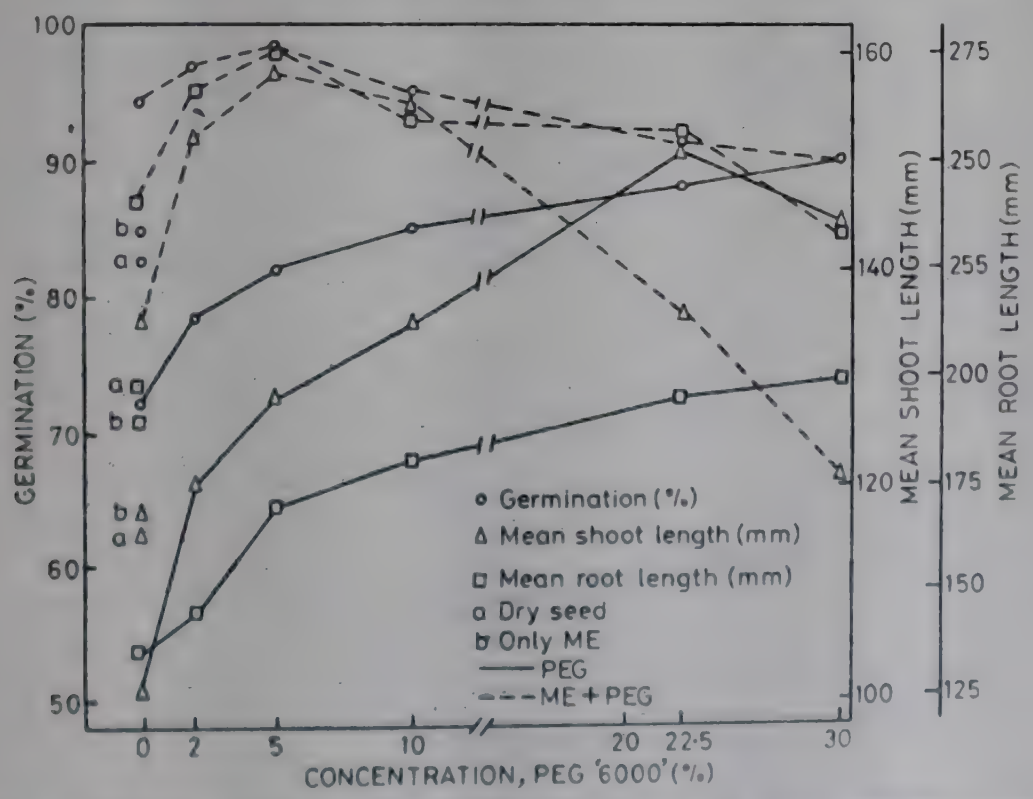

Figure 2 Effect of osmoconditioning and moisture equilibration on germination percentage and seedling vigour of soybean seeds following treatment

length showed a marked decline at 22.5 and 30% PEG.

In order to study the effect of treatments during subsequent storage the treated seeds were dried back and subjected to accelerated ageing for 6 days at 90% RH and 40°C to obtain results in a short period. The osmotically treated seeds now showed much lower germination and poor seedling growth as compared to the untreated control (figure 3). Seed soaking or moisture equilibration followed by drying was also found to be deleterious for vigour and viability under accelerated ageing. On the other hand, treatment of pre-equilibrated seeds with different concentrations of PEG proved beneficial in retaining higher germinability and better seedling growth even after drying and storage. ME followed by soaking in water (0% PEG), showed appreciable effect in the maintenance of vigour. Maximum beneficial effect was observed when pre-equilibrated seeds were treated with 2–5% PEG. Higher concentrations of PEG (22.5–30%) proved detrimental to the maintenance of viability.

Release of electrolytes by leaching during seed imbibition was highest in seeds which were not preconditioned. Such leakage was considerably reduced following equilibration or osmoconditioning of seed (table 1). Leakage was minimum at higher concentrations (22.5 and 30%) of PEG; at lower (2–5%) PEG concentrations, leakage could be reduced effectively if the seeds were equilibrated with a saturated atmosphere prior to

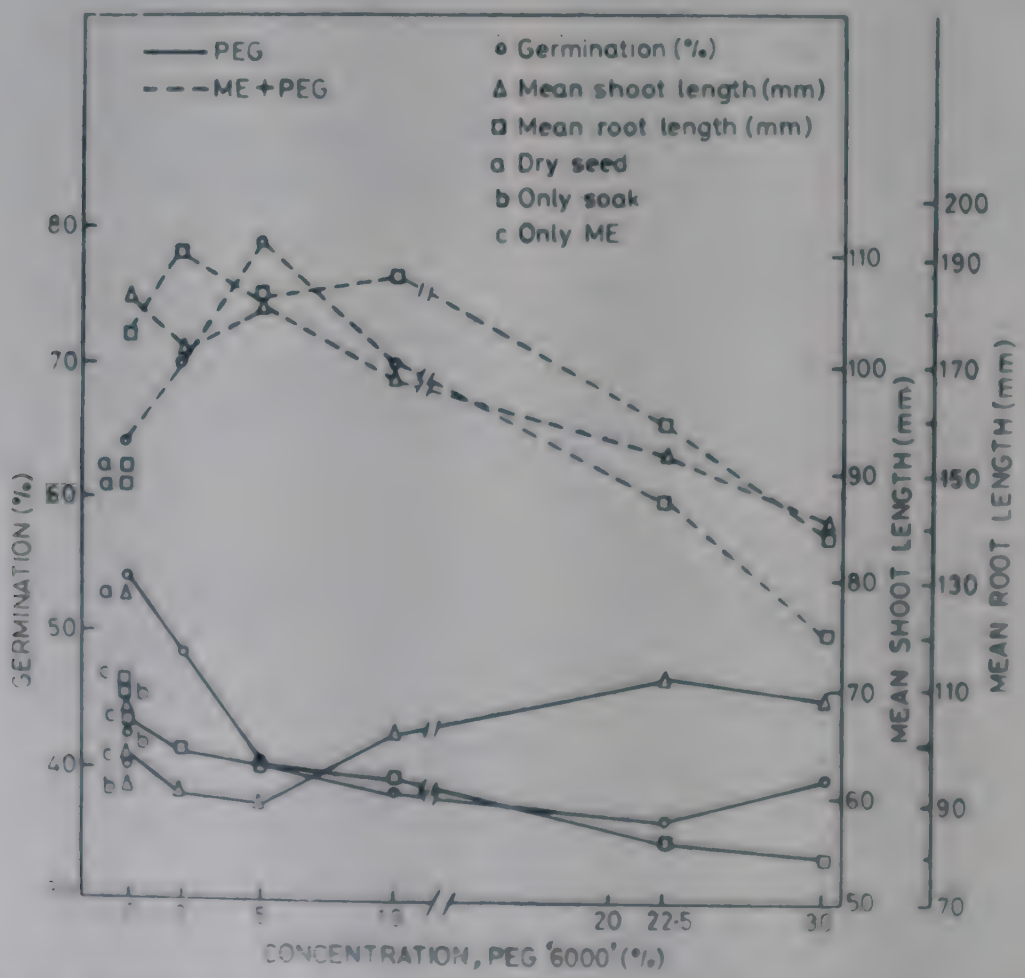

Figure 3 Effect of osmoconditioning and moisture equilibration followed by drying on viability of soybean seeds stored for 6 days at 90% RH and 40°C

Table 1 *Effect of moisture equilibration and osmoconditioning on membrane integrity during soaking. Forty seeds soaked in 20 ml of water/PEG-6000 in each of four replicates for 2 hours and transferred to water for an additional one hour. Seed equilibrated in a water-saturated atmosphere at 28°C for 48 hrs*

Treatments (Soaking)	Conductivity of seed leachate, μmhos			
	Non-equilibrated		Equilibrated	
	Initial soaking for 2 hrs	Further soaking in water for 1 hr	Initial soaking for 2 hrs	Further soaking in water for 1 hr
Water	772	124	565	200
PEG (%)				
2.0	717	143	303	209
5.0	598	109	203	200
10.0	367	112	174	208
22.5	227	146	107	210
30.0	160	146	62	230

osmoconditioning. The latter also gave the most beneficial effect on prolonging storability of seeds. It appears that maximum leakage of electrolytes from seeds occurs during the first two hours, since an additional hour of soaking in distilled water following a 2-hr treatment produced no appreciable effect.

The moisture uptake of PEG-treated seeds (both nonequilibrated and moisture-equilibrated) after 2 hr of soaking was less than the untreated water soaked seeds especially at higher concentration of PEG (table 2). Moisture equilibrated seeds had a much higher moisture content than nonequilibrated seeds without or with PEG after initial 2 hr soaking. However, after 1 hr of further soaking in distilled water the moisture content of untreated and PEG treated seeds attained almost the same level (49.5 to 51.4 in nonequilibrated; 57.1 to 60.7 in moisture equilibrated seeds). Again, the moisture equilibrated seeds treated with or without PEG, showed a much higher moisture content than nonequilibrated seeds. It may be mentioned here that the preequilibrated seeds when placed in higher concentration of PEG, absorbed less moisture; however when these were again placed in distilled water moisture uptake was quite rapid as compared to seeds which were equilibrated, then placed in lower concentration of PEG followed by soaking in distilled water, to reach the final moisture content.

Discussion

The results show that the treatments that ensured slow hydration of seeds improved seed germination and seedling growth. Moisture equilibration (ME) and polyethyleneglycol treatment either alone (PEG) or following moisture equilibration (ME-PEG), were better than water-soaking alone (WS), with ME-PEG-WS having the slowest imbibition and thereby the greatest improvement. In other words, the imbibitional injury to seed and/or seed structure during soaking directly in water is due to rapid entry of water (Powell & Matthews 1978). When seeds or their embryonic axes are placed for soaking in water, rate of imbibition is very rapid and injury is caused due to anoxia (Orphanos & Heydecker 1968), sharp decrease in enzyme activity (Duke & Kekefuda 1981) and destructive changes in ultrastructure (Powell & Matthews 1979). In fact, Woodstock and Taylorson (1981) have shown that just one-hour soaking of soybean seeds can markedly inhibit germination and seedling growth. Our results also confirm these observations.

Osmotic treatment in the PEG reduces imbibitional or chilling injury in legume seeds (Tulley et al. 1981). In low vigour seeds of soybean, soaking of seeds in 30% PEG could effectively avoid soaking

Table 2 *Moisture changes in (dry and moisture equilibrated) seed during osmoconditioning. Dry untreated seed did not receive any humidification; moisture equilibration done in a water-saturated atmosphere for 48 hours. Data presented in parentheses show the increase in moisture content (% w.wt) over untreated control. Other details same as in table 1*

Treatments (Hydration)	Non-equilibrated, % MC, w. wt.			Moisture-equilibrated, % MC, w.wt.		
	Initial	After 2 hour soaking	Further 1 hour soaking	Initial	After 2 hour soaking	Further 1 hour soaking
Untreated	9.1			—		
Moisture equilibrated	—			21.1		
Water soaked		44.3 (35.2)	51.4 (42.3)		54.0 (45.0)	57.4 (48.3)
Soaked in PEG (%)						
2.0		42.1 (33.0)	50.9 (41.8)		55.0 (46.9)	60.0 (50.9)
5.0		41.1 (32.0)	51.0 (41.9)		56.1 (47.0)	60.7 (51.6)
10.0		38.5 (29.5)	49.9 (40.8)		53.3 (44.2)	59.1 (50.0)
22.5		34.4 (25.3)	49.5 (40.4)		47.2 (38.1)	57.4 (48.3)
30.0		31.2 (22.1)	50.4 (41.3)		46.3 (37.2)	57.1 (48.1)

injury and improve germinability and vigour of treated seeds (Woodstock & Taylorson 1981). Bramlage et al. (1977) have reported that humidification of soybean seeds before imbibition reduced leakage and chilling injury during soaking. Saha and Basu (1982, 1984) have shown that soaking of PEG-treated seeds would improve immediate germinability as well as storability in soybean. In the present investigation it has been found that the treatment ME-PEG-WS is most promising in improving immediate germinability as well as storability of a seed lot. Beneficial effect of moisture equilibration in reducing membrane leakage was reported earlier by Rudrapal and Basu (1979) and Dey and Basu (1982) who suggested a control of free radical-mechanism induced membrane damage in this treatment. Basu and Dasgupta (1978) have shown that the moisture content of seeds attained during moisture equilibration alone (20–25%) was sufficient to prevent the damage due to free radical reactions in wheat seeds. But in soybean seeds only moisture equilibration-drying treatment failed to maintain viability under storage.

The higher moisture content of seed reached during the ME-PEG-WS treatment, unaccompanied by any severe leakage loss, may be necessary for counteracting the free radical pathology or lipid autooxidation damage during storage. Such treatments may also initiate or improve repair mechanism and prevent deterioration (Villiers & Edgcumbe 1975, Hegarty 1978).

Thus moisture equilibration followed by osmoconditioning which showed smooth

entry of water into the seed unaccompanied by soaking injury to membrane, possibly without affecting membrane reorganisation, considerably improved immediate germinability and retained viability of the soybean seed for a longer period.

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Proc. Indian natn. Sci. Acad. B51 No. 4 pp 522-527 (1985)

Photoproduction of Ammonia by Cyanobacteria

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(Received 16 April 1985)

Nitrogen-fixing cyanobacteria have the ability to convert solar energy into chemical energy-ammonia, at the expense of sunlight and water alone. Cultures of the cyanobacteria *Gloeocapsa* and *Mastigocladus laminosus* excrete newly fixed ammonia when treated with L-methionine-DL-sulphoximine (MSX). This inhibitor, a glutamate-analogue, induces ammonia excretion by inhibiting the activity of glutamine synthetase (GS), the key enzyme in ammonia assimilation. A single addition of 10 μM MSX induces ammonia excretion for 6 days in *M. laminosus* and 15 days in *Gloeocapsa*. While the GS of *Gloeocapsa* gets inactivated irreversibly by MSX-treatment, that of *M. laminosus* recovers from the 5th day. In addition, *M. laminosus* excretes ammonia when incubated in light or dark and also on treatment with combined nitrogen. *Gloeocapsa* excretes ammonia only in the presence of light. This process of photo-production of ammonia by these cyanobacteria offers great promise for their use as biofertilizers in rice paddies.

Key Words: Cyanobacteria, Photoproduction, Ammonia, L-methionine-DL-sulphoximine, Biofertilizer, Glutamine synthetase-inhibition

Introduction

Nitrogen-fixing cyanobacteria have the unique ability to fix dinitrogen into ammonia at the expense of sunlight and water alone. The fixed ammonium is the primary source of all nitrogenous fertilizers. Except symbiotic nitrogen-fixing cyanobacteria, in most of the free-living, nitrogen-fixing cyanobacteria, ammonia is never excreted in the medium under normal physiological conditions and most of the fixed nitrogen is available only after cell death. Cells of *Nostoc* and *Anabaena azollae* growing symbiotically in the lichen *Peltigera canina* and the water fern *Azolla* respectively, release a high percentage of the nitrogen that they fix to the Symbiont (Stewart et al. 1980). This suggests that cyanobacteria can tolerate to some extent, the release of nitrogen from the cells. Such a release is also sought to be compensated by an increase in nitrogenase activity. The mechanism by which nitrogenase activity is increased is still not clear. However, two possibilities appear feasible. First, the repression of

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nitrogenase by combined nitrogen apparently acting through a product of GS action (Stewart & Rowell 1975) is removed. Secondly, inhibition of the primary ammonia assimilating enzyme GS causes the release of freshly formed ammonia out of the cells and creates a nitrogen starvation condition. This drives the organism towards anoxygenic photosynthesis, which is conducive for nitrogenase action.

Release of ammonia by cells treated with L-methionine-DL-sulphoximine (MSX) has been reported among cyanobacterial species (Stewart & Rowell 1975, Ramos et al. 1982a). MSX, a glutamate-analogue, induces ammonia excretion by inhibiting glutamine synthetase (GS), the key ammonia assimilating enzyme. This paper reports the sustained production of ammonia by two species of nitrogen-fixing cyanobacteria—*Gloeocapsa* sp., and *Mastigocladus laminosus* Cohn, and discusses the feasibility of transferring this technology to the rice field.

Materials and Methods

Organisms and Growth Conditions: *Mastigocladus laminosus* Cohn. (CCAP 1447/1) was grown at 37 ± 2°C under continuous illumination (40W, Cool daylight) of 3000 lux. *Gloeocapsa* sp. (IUCC, LB 795) was grown at 25 ± 1°C and illuminated with cool white fluorescent lamps at an intensity of 500 lux and 12 : 12 hr LD. Both cyanobacteria were grown as semi-continuous cultures in Allen and Arnon's (1955) medium free of combined nitrogen or amended with 20 mM KNO_3, and aerated continuously with a stream of sterile humidified air.

Ammonia production experiments: Cells containing 15–30 μg Chl/ml were harvested by centrifugation, washed with fresh culture medium and resuspended in the same medium to a density of 5–6 μg Chl/ml. MSX was added to the cell suspensions at the final concentrations of 10 and 100 μM and incubated under standard culture conditions in light or in dark (wrapped in aluminium foil). MSX was prepared freshly in distilled water as required and sterilized by filtration.

Enzyme extraction: Cell-free extracts were prepared at 4°C after harvesting the cyanobacteria by centrifugation, washing twice and resuspending in 10 mM Imidazole-HCl, *p*H 7.5. Cells were then disrupted in an ice-bath by sonic oscillation in MSE 150W Ultrasonic Disintegrator and debris removed by centrifugation at 20,000 × g for 30 min.

Enzyme assays: The transferase activity of glutamine synthetase (E.C 6.3.1.2) was performed according to Shapiro and Stadtman (1970) at *p*H 7.5. The Mn^{2+}-concentration was reduced 10-fold to 0.3 mM to prevent $Mn_3(AsO_4)_2$ precipitation.

Analytical methods: Ammonia was estimated by the phenol-hypochlorite method of Solorzano (1969). Protein was determined according to Lowry et al. (1951) after pretreating the extract with 10% (W/V) trichloroacetic acid. Bovine serum albumin served as standard.

Results

Nitrogen-grown, light-incubated cultures of *Gloeocapsa* and *M. laminosus* possess high levels of glutamine synthetase. On treatment with MSX, GS activity decreases. In *Gloeocapsa*, MSX acts as an irreversible inhibitor and GS activity does not recover even on prolonged incubation (figure 1). In *M. laminosus*, on the other hand, following the initial lowering of activity, GS activity starts increasing from the 5th day (figure 2). MSX, even at 10 μM brings about nearly total inhibition of GS in both cyanobacteria (table 1).

Under normal culture conditions, ammonia is not excreted into the medium by both *M. laminosus* and *Gloeocapsa*. Filaments of *M. laminosus* excrete ammonia, 4 hr after the addition of MSX (figure 3); excretion

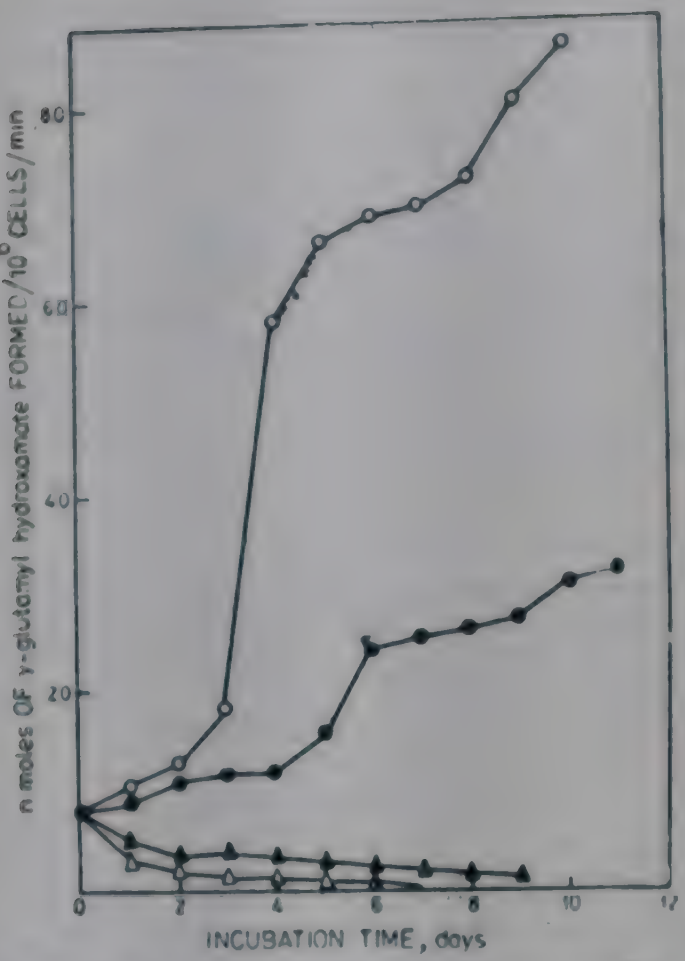

Figure 1 GS activity of N_2-grown *Gloeocapsa* incubated with (*triangle*) or without (*circle*) or 10μM MSx under light (*open symbols*) or dark (*closed symbols*).

Table 1 *Percentage inhibition of GS by 10 μM MSX*

Day	*M. laminosus*		*Gloeocapsa*	
	Light	Dark	Light	Dark
1	32	24	80	72
2	66	40	88	78
3	89	67	94	86
4	94	69	99	88
5	94	78	99	92
6	85	72	100	94
7	—	—	100	96
8	—	—	100	96

Both cyanobacteria were grown in N-free medium
Percentage GS inhibition was computed with reference to MSX-untreated cultures

continues for 8 days when treated with 10 μM MSX. The period of ammonia excretion can be prolonged (to 13 days) by the addition of 100 μM MSX. In the former condition, the average production is $\pm$ 8 nmoles NH_3/μg Chl/day while in the latter the rate is nearly five-fold more. Maximum ammonia production is noticed on the 4th day when GS gets inactivated to its maximum. The rate of ammonia release slows down thereafter coinciding with the recovery of GS activity. MSX-treated cultures of *Gloeocapsa*

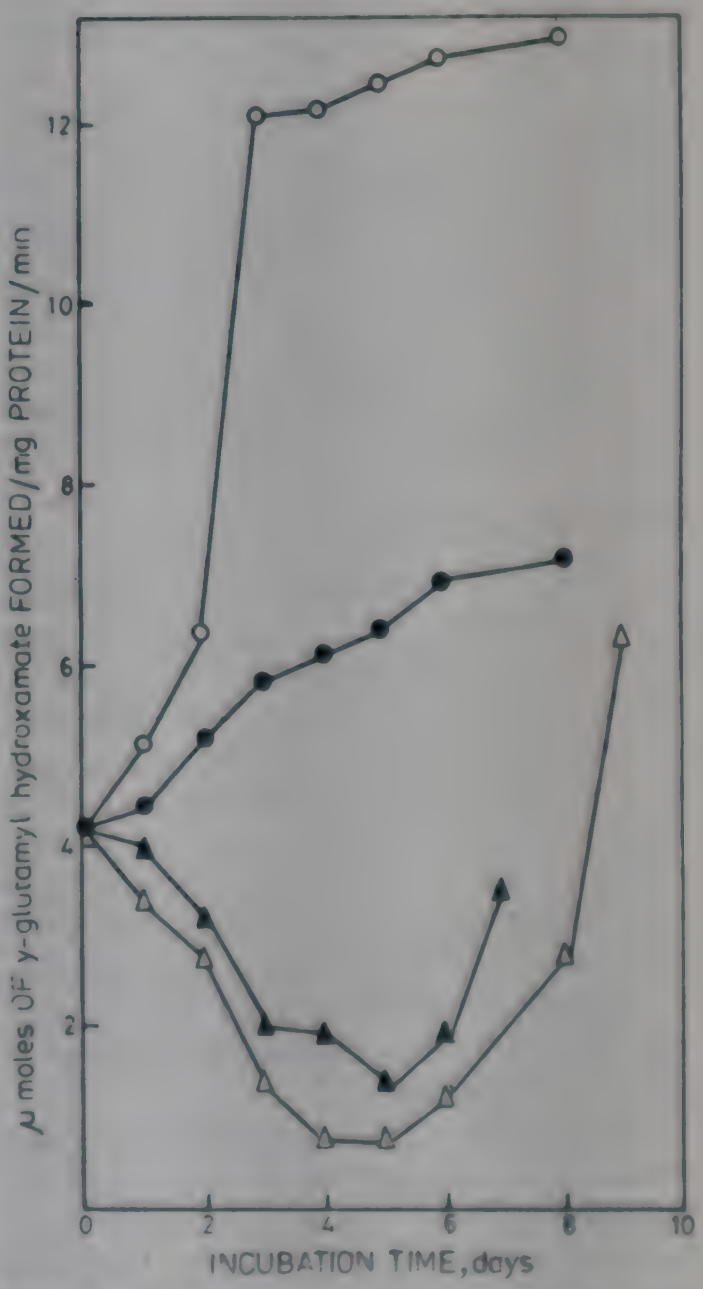

Figure 2 GS activity of N_2-grown *M. laminosus* incubated with (*triangle*) or without (*circle*) 10 μM MSx under light (*open symbols*) or dark (*closed symbols*).

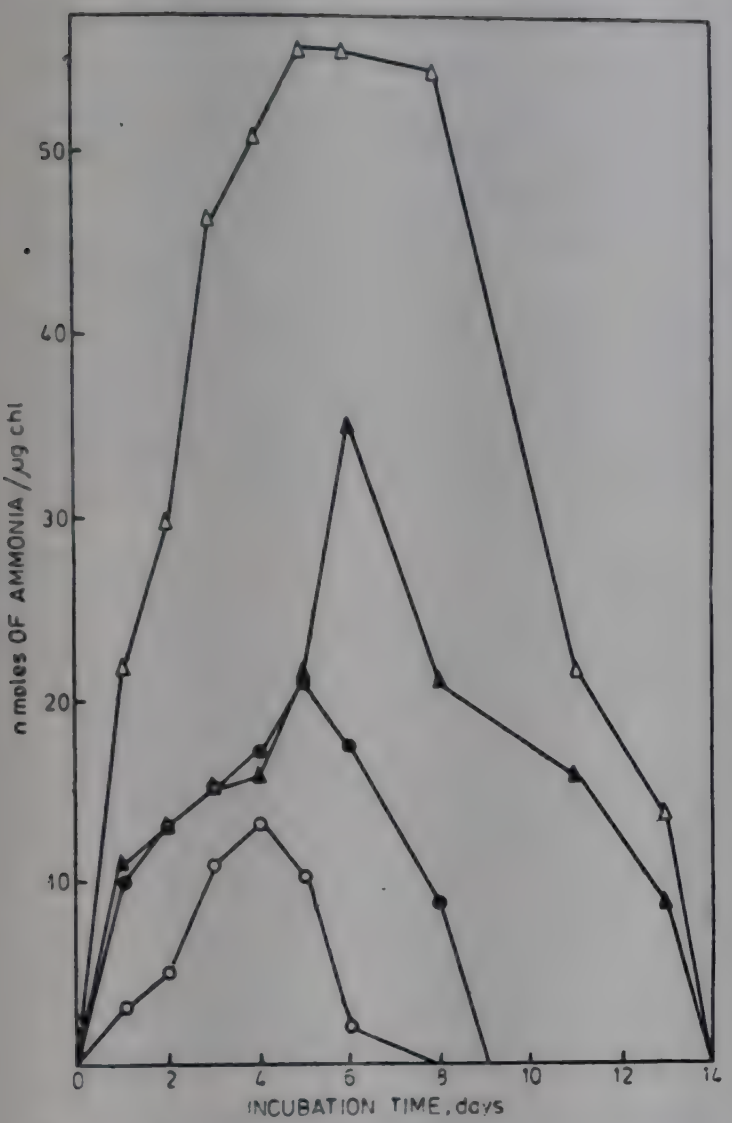

Figure 3 Excretion of ammonia by N_2-grown *M. laminosus* incubated with 10 μM (*circle*) or 100 μM (*triangle*) MSx in light (*open symbols*) or dark (*closed symbols*).

excrete ammonia only from the 3rd day (figure 4). Excretion continues for 15 days with peak production on the 6th day when GS gets totally inactivated, with an average production of 220 nmoles NH_3/μg Chl/day.

GS activity is lowered by 50% and 65% in *M. laminosus* and *Gloeocapsa* respectively (figure 1 and 2) on transfer of light-grown cells to the dark. The inactivation by MSX is also lower than in light-grown cells. The overall pattern of GS inhibition and ammonia production are otherwise similar. In *M. laminosus*, ammonia excretion in dark appears to be independent of MSX concentration, the average being 15 and 17 mnoles NH_3/μg Chl/day at 10 and 100 μM MSX respectively. In *Gloeocapsa*, ammonia production in dark is negligible in comparison to light-incubated cells.

When these nitrogen-fixing cyanobacteria are grown on combined nitrogen (20mM NO_3^-), they show comparable rates of GS activity (figure 5 and 6) and nearly the same amount of ammonia production (in *Gloeocapsa*). In *M. laminosus*, the cultures show 3-fold and 5-fold increase in ammonia production when treated with 10 and 100 μM MSX respectively (figure 5).

Discussion

Inhibition of GS and excretion of ammonia in response to MSX-treatment have been reported in bacteria (Brenchley 1973, Weare & Shanmugam 1976) and in cyanobacteria like *Anabaena cylindrica* (Stewart & Rowell

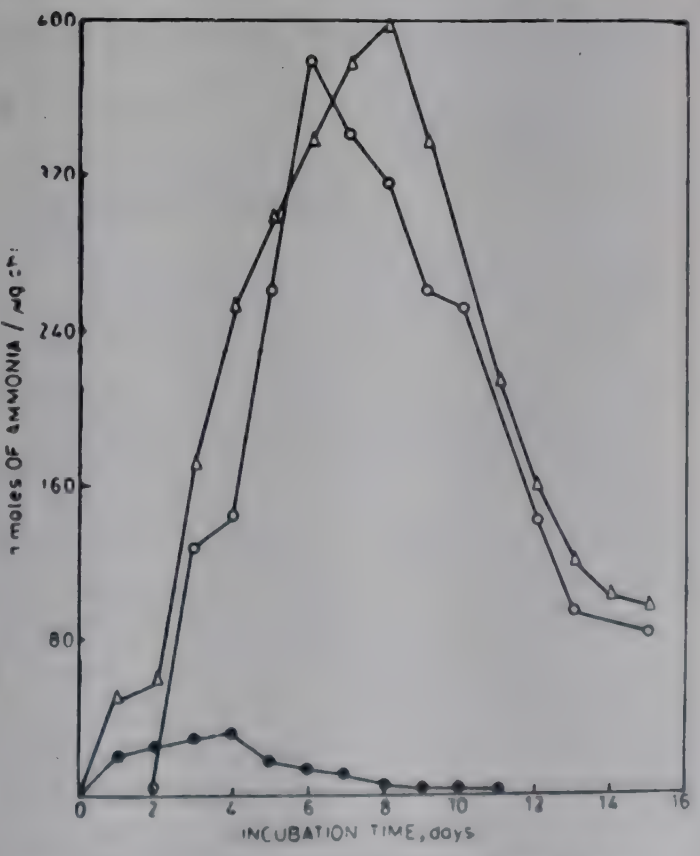

Figure 4 Excretion of ammonia by N_2-grown (*circle*) or NO_3^--grown (*triangle*) *Gloeocapsa* incubated in light (*open symbols*) or dark (*closed symbols*) with 10 μM MSx.

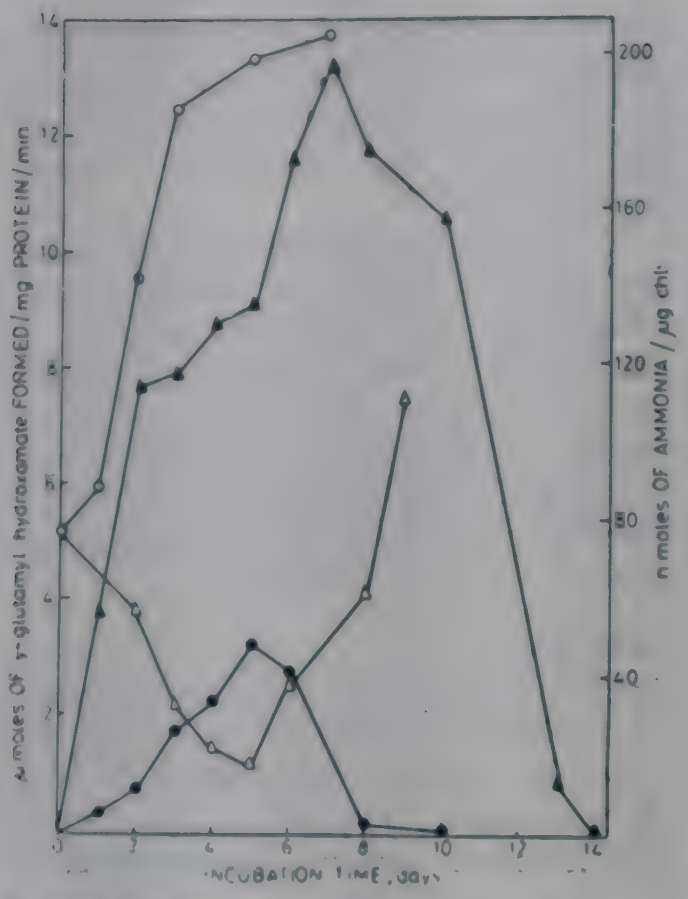

Figure 5 GS activity (*open symbols*) and ammonia excretion (*closed symbols*) by NO_3^--grown *M. laminosus* incubated in light without (o) or woth 10 µM (△●) or 100 µM (▲) MSx.

1975) and *Anacystis nidulans* (Ramos et al. 1982a). Our results show that the nitrogen-fixing cyanobacteria, *M. laminosus* and *Gloeocapsa* are capable of excreting ammonia when treated with MSX. In fact, these cyanobacteria are superior to the other two cyanobacteria mentioned above in being able to produce ammonia continuously for prolonged periods of time (6 days by *M. laminosus* and 15 days by *Gloeocapsa*) in response to a single addition of 10 µM MSX. It is interesting that while MSX acts as a reversible inhibitor in *M. laminosus*, it inactivates GS irreversibly in *Gloeocapsa*. However, GS of the other unicellular cyano Bacterium *A. nidulans* is reversibly inhibited by MSX (Ramos et al. 1982b).

Nitrogen-fixing cyanobacteria are known to fluorish in rice fields where they contribute to the nitrogen economy of the soil. Increase in grain yield by 10–20% has been reported in response to "algalisation" without any added chemical nitrogen fertilizer. The algal technology developed at the IARI has been shown to be capable of providing 20–30 kg of biologically fixed nitrogen per hectare of rice per season (Venkataraman 1972, 1981). This quantity of nitrogen is inadequate to maintain the rice crop and has to be supplemented with other nitrogenous fertilizers. Our results show that MSX treatment leads to the release of substantial

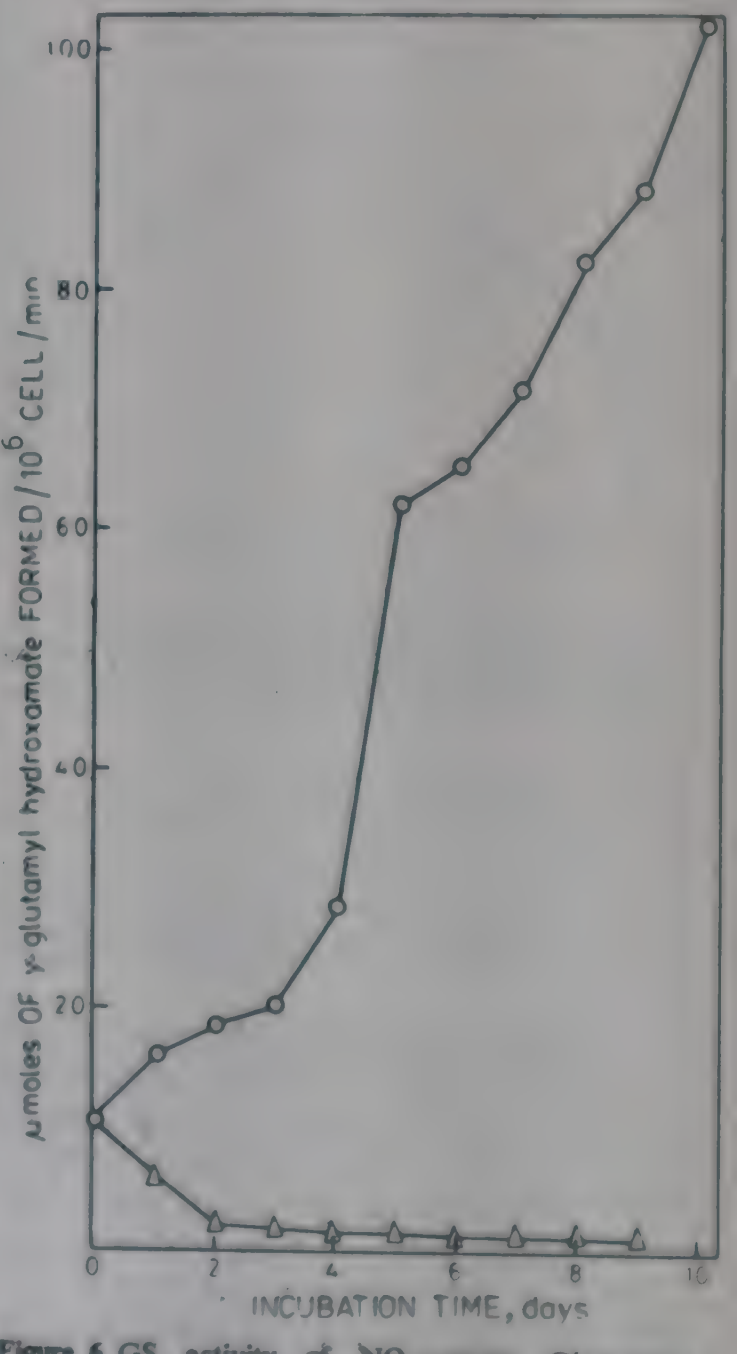

Figure 6 GS activity of NO_3^--grown *Gloeocapsa* incubated in light with (△) or without (o) 10 µM MSx.

quantities of ammonia, a form of nitrogen easily assimilated by higher plants. *M. laminosus* is more promising since its GS recovers once the MSX-effect ceases. This would enable the cyanobacteria to remain viable and by properly spacing the MSX treatments, rich benefits could be achieved.

The full potential of the use of these cyanobacteria as biofertilizers lies in the fact that they should be able to release freshly fixed ammonia as the symbiotic rhizobia and *Anabaena azollae* do. Hitherto it was believed that the cyanobacteria are capable of contributing the fixed nitrogen only on their death. But the technique of inhibiting the primary ammonia assimilating enzyme, temporarily in *M. laminosus* offers an excellent scope for the exploitation of such cyanobacteria as good biofertilizers, generating valuable nitrogen fertilizer *in situ*, using solar energy alone. This organism being a thermotolerant species is ideal for the tropical region.

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Biological Causes Governing Head Mold of Pearl Millet

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(Received 30 April 1984; after revision 7 October 1985)

The association of head mold fungi with the seeds of pearl millet resulted in seed rot, seedling blight and damping-off. The spores from such infected parts become air-borne which get deposited on plant parts including the earhead. The persistence of the anther lobes up to the grain filling stage of earhead growth under humid conditions is the major pre-disposing factor for head mold infection.

Key Words: *Pennisetum americanum*, **Earhead infection, Persistent anther lobes, Pollen, Fungal spore stimulants**

Introduction

Of the various organisms attacking pearl millet (*Pennisetum americanum* (L.) Leeke = *P. typhoides* (Burm.) Stapf and C.E. Hubb.), fungi account for 94% (Shetty et al. 1982) and a major number of these fungi are seed-borne. About 33 diverse types of fungi are known to be associated with pearl millet seed samples of southern India and they are known to reduce the quality and germination of the seeds (Raveesha 1982). Fungi like *Drechslera setariae*, *Cladosporium cladosporioides*, *Phoma exigua* and *Fusarium* spp are reported to be seed-borne and pathogenic to pearl millet (Ramakrishnan 1963, Mathur et al. 1973, Shetty et al. 1982, Raveesha 1982). But no information is available on the conditions which favour association of these fungi with seeds. This paper elucidates the factors which help in establishment of fungi on earheads/seeds and its implications.

Materials and Methods

Effect of Pathogenic Seed-borne Fungi on Germination

Seeds collected from four different pearl millet growing areas were screened for mycoflora by employing standard blotter test (ISTA 1966). The sub-samples of seeds were sown in field. Six days after sowing, number of seedlings that emerged were counted. The ungerminated seeds were recovered from soil, washed in sterile water to separate the soil debris and incubated on sterile wet blotter at 22 ± 1°C with 12/12 hr cycle of Near Ultraviolet (NUV) light and darkness. After four days, observation was made to record the specific fungi responsible for seed rot.

Field Observations

Seeds of pearl millet (cv. IP 7042) were sown in a 15×15 m plot. The plants were inspected at regular intervals till

harvest for any disease symptoms other than downy mildew, ergot and rust. Normal agronomic practices were followed.

Earhead Infection

Observations were made on the time required for the completion of different stages of earhead growth and the causes of earhead infection.

Survey of the fungal flora over pearl millet plants: A vertical cylinder trap was set at the height of the crop and in the centre of the plot. Procedures of Ramalingam (1968) were followed in the preparation of vertical cylindrical trap. A 20×20 mm cellophane strip smeared with vaseline was wound round a 7 mm diameter glass rod (15 cm long) and mounted on a specially designed stand. The traps were replaced daily by 10 AM. The cellophane strips were observed under microscope after mounting in glycerine with 0.5% cotton blue.

Mycoflora of persistent anther lobes and dead parts of the plant: The persistent anther lobes were carefully separated from the earheads and incubated in moist chambers for four days under NUV/darkness (12/12 hr) cycles at 22±1°C to record the associated fungi.

The basal senescent leaves with necrotic lesions and mulch formed by the plant refuse were surface sterilised using 2% NaOCl (10 min), washed in distilled water, incubated on sterile wet blotters for 48 hr under 12/12 hr cycle of NUV/darkness at 22±1°C and observed for the associated fungi.

Visual symptoms of earhead/seed infections: The symptoms of head mold infections were carefully observed and recorded. The seeds collected from head mold-infected earheads were subjected to standard blotter test to study the mycoflora associated with them. A sub-sample of the seed was subjected to maceration test. In 200 ml of 10% KOH containing 1% trypan blue, 25 g seeds were soaked for 24 hr. The seeds were thoroughly washed in running water to remove excess of alkali and then transferred to lactophenol. The material was gently warmed to remove excess of stain and then observed under stereobinocular microscope.

Interaction of pollen and fungal spores

Pure culture of *D. setariae* and *C. cladosporioides* were isolated from the anther lobes of IP 7042 cultivar of pearl millet. For collecting the pollen, paper bags were inserted over pearl millet earheads which were about to liberate the pollen at 6 PM. The next morning by 8 AM the bags were recovered. Immediately the contents of the paper bags were sieved through cheese cloth to separate the pollen from dehisced anther lobes. The pollen was stored in water proof glass containers at 5°C. Conidial suspensions of *D. setariae* and *C. cladosporioides* were prepared in 10 ml sterile water, to which 5 mg, 10 mg and 20 mg pollen was added. Five ml of each concentration was spread on water agar (1.5%) in sterile Petri plates. These plates were incubated at 22±1°C in darkness. After 24 hr of incubation, the germination of conidia of the fungi in pollen suspensions was recorded. The conidial suspensions without pollen served as control.

Observations

Effect of Seed-borne Fungi on Germination

Seeds of Sample No. 1 which were heavily infected by *Drechslera setariae*, *Cladosporium cladosporioides* and *Phoma exigua* (table 1) showed least germination both under field and laboratory conditions (table 2). In sample No. 2, in addition to these fungi, *Aspergillus* and *Penicillium* species also contributed for the poor germination and seedling rot. Most of the rotted seeds of sample No. 3 were colonised by *C. cladosporioides*

Table 1 *Seed mycoflora of pearl millet seed collected from the head mold occurring fields*

Fungus	% incidence in sample No.			
	1	2	3	4
Aspergillus flavus	—	43.0	—	2.5
A. candidus	—	3.0	—	0.5
A. niger	—	5.5	0.5	7.5
A. terreus	—	18.5	2.0	—
Alternaria alternata	12.0	3.5	7.0	—
Cladosporium cladosporioides	32.0	2.5	39.0	0.5
Cephalosporium acremonium	2.0	—	—	—
Curvularia penniseti	22.0	15.0	—	—
Drechslera setariae	75.0	3.5	—	5.0
D. tetramera	19.0	6.5	—	—
Fusarium equiseti	6.0	—	—	—
F. moniliforme	—	—	—	0.5
F. semitectum	—	—	—	0.5
F. solani	—	—	—	1.0
Penicillium sp.	—	25.5	3.0	—
Phoma exigua	73.0	4.5	43.5	9.0
Rhizopus arrhizus	—	4.5	3.5	3.5
Trichoderma viride	—	—	0.5	2.5

Table 2 *Comparison of seed germination on wet blotter and field soil and per cent incidence of fungi on ungerminated and rotted seeds*

Sample No.	Germination under field condition (%)	Germination under laboratory condition (%)	Pre-emergence damping-off in field soil				
			Drechslera setariae	*C. Caldosporioides*	*Phoma exigua*	*Aspergillus*	Other fungi
1	9.0	11.0	42.0	9.0	18.0	2.0	29.0
2	36.5	29.5	5.0	0.0	2.0	47.0	49.0
3	87.0	84.0	0.0	26.0	29.0	1.0	41.0
4	79.0	82.0	2.0	0.0	11.0	38.0	48.0

The data are based on 400 seeds tested per sample

and *P. exigua*. In sample No. 4, *Aspergillus* and *P. exigua* were responsible for germination failure (table 2).

Field Observation

The first pathogen to express at the seedling stage of the plant was *D. setariae*. More than 20% of the seedlings were showing seedling blight and damping off. No leaf spots were observed on the seedlings but the leaves were blighted throughout their length. Such leaves upon incubation showed good growth of *D. setariae*. The fresh growth between 2–6 weeks was almost free from *D. setariae* infection. However, after six weeks, necrotic, tan-buff coloured, 3–7 mm long spots appeared on the leaves. Upon incubation on wet blotters, *D. setariae* was isolated from such spots. Sometimes the spots coalesced resulting in irregular necrotic lesions. Necrotic, tan-buff coloured spots also appeared on culm. The pathogen was also isolated from the senescent second and third leaf of the plant. At the time of earhead maturity, thick growth of *D. setariae* was observed on the earheads. When the persistent anther lobes and the earheads were incubated on wet blotters, thick growth of *D. setariae* was observed on the anther lobes and seeds.

Cladosporium cladosporioides which was found to cause seed rotting, did not produce severe blighting of the leaves. However, the senescent leaves of the plants carried this fungus. At the time of crop maturity, black or brown or dark brown restricted coloured necrotic spots measuring 2–3 mm in diameter were observed on the topmost leaves which on incubation showed colonies of *C. cladosporioides*. This fungus was also observed to be one of the components of earhead infection and was isolated both from the remnants of the anther lobes and developing seeds.

Phoma exigua caused only seed rotting. The fungus was not found on green leaves. However, the basal senescent leaves showed the presence of embedded pycnidia of *Phoma* when incubated. From none of the necrotic lesions present on the green parts of the plants, *Phoma* was isolated.

Earhead Infections

The duration for the completion of different stages of earhead growth were recorded. It was noted that in most of the plants, the anther lobes were attached to the earheads even when the kernels were at the hard dough stage.

Survey of the fungal flora over pearl millet plants: The microscopic observation of the cellotapes of cylinder traps revealed the presence of conidia of *D. setariae*, *D. tetramera*, *D. rostrata*, *C. cladosporioides*, *Curvularia penniseti*, *Fusarium* spp. and *Alternaria* spp.

Mycoflora of anther remnants and dead parts of the plants: The mycoflora of anther lobes retained on the earheads after anthesis has been presented in table 3. It was

Table 3 *Mycoflora of anther lobes of pearl millet*

Fungus	Sample No. / % incidence				Average
	1	2	3	4	
Drechslera setariae	93	100	96	98	96.92
Cladosporium cladosporioides	93	100	98	91	95.50
Phoma exigua	43	32	56	29	40.00
Fusarium spp.	23	28	33	22	26.50
Nigrospora sp.	8	0	7	11	6.50
Penicillium sp.	3	4	0	3	2.50

observed that *D. setariae* and *C. cladosporioides* were the frequent colonisers of the anther lobes. *Phoma* spp., *Fusarium* spp., *Nigrospora* sp. and *Penicillium* were also recorded. Invariably the anther lobes were colonised by more than one type of fungi and more often than not, *D. setariae* and *C. cladosporioides* were found to grow together on a single anther lobe.

The basal dry leaves of plant harboured fungi like *D. setariae*, *C. cladosporioides*, *Phoma exigua*, *Penicillium* sp. and a wide variety of other fungi. Plant debris like decaying culms and glumes were also found to carry the said fungi.

Visual symptoms of earhead infection: Under humid conditions, a thick fungal growth was observed on the earheads. When the head mold infected earheads were observed under stereobinocular microscope, *D. setariae*, *C. cladosporioides*, *Fusarium* spp. and *Phoma exigua* were found colonising the anther lobes, which later invaded the developing grains through the region of attachment of the anther lobes. *Fusarium* spp. was found to grow between the spaces of developing seeds after growing on the anther lobes. Histopathological preparations substantiated this observation. When the seeds from moldy earheads were subjected to standard blotter test, as much as 85% of seeds were found to carry *D. setariae*, 26.5% *C. cladosporioides*, 25.3% *Phoma* sp., 4% *Penicillium* spp. and 1.5% *Alternaria* spp. The germination of the seeds was as low as 25.5%. Multiple infection was very common. Upon maceration, fungal mycelium was found in the embryo, endosperm and pericarp of the seed. Specific effect of *Cladosporium* was seed abortion. Under severe infection, the pathogen colonised the whole earhead and as a result no grains were formed.

Interaction of Pollen and Fungal Spores

It was significant to note that the pollen enhanced the germination of conidia of *C. cladosporioides* and *D. setariae*. The germination of *C. cladosporioides* was enhanced by five times in the presence of pollen at the rate of 10 mg/10 ml of conidial suspension (figure 1). Pollen enhanced the conidial germination of *D. setariae* also.

Discussion

Several seed-borne fungi are known to be responsible for seed rot, seedling blight and damping off of pearl millet (Wells & Wingstead 1965, Mathur et al. 1973, Sharma & Basu Chaudhari 1975, Randhava & Aulakh 1980, Shetty et al. 1982). In the present investigation, the role of *D. setariae*, *C. cladosporioides* and *P. exigua* and a few

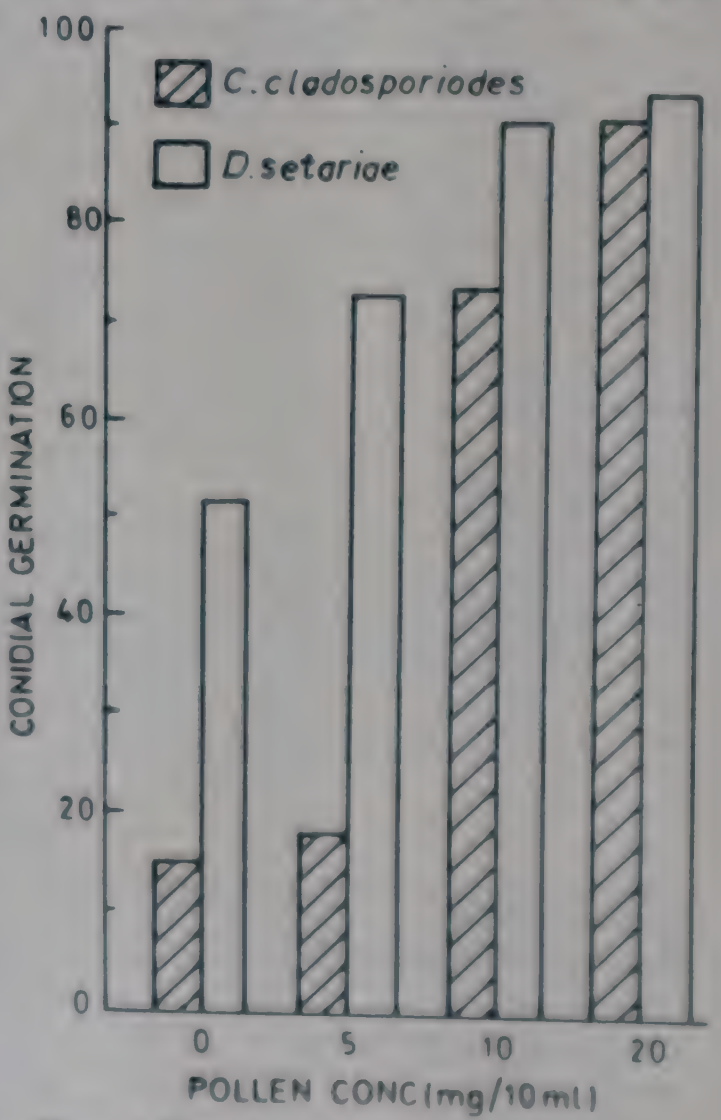

Figure 1 Effect of pearl millet pollen on conidial germination of *C. cladosporioides* and *D. setariae*

other fungi in causing seed rot have been demonstrated.

As observed by Luttrell (1954), Wells and Burton (1967) and Shetty et al. (1982), pearl millet plants are susceptible to infection by *D. setariae* at two growth stages viz., seedling and senescence stage. The nature of symptoms of the disease at these two susceptible stages have been described in this study. Similarly the nature of disease incidence due to *C. cladosporioides* has also been dealt with.

Very little work has been done on head mold problem in pearl millet. Wiehe (1953) noticed that *Cladosporium herbarum* could cause moldiness of the ears and this resulted in uneven development of the grains. Onesirosan (1975) reported that *Fusarium* spp. are responsible for head mold of pearl millet in Southern Nigeria. Mathur et al. (1960) reported the blackening of grains due to *Curvularia penniseti*. The present investigations revealed that *D. setariae*, *C. cladosporioides*, *Phoma exigua*, *Alternaria* spp. and *Fusarium* sp. are the major fungal components of moldy grains. These fungi also cause head mold of sorghum (Bhagawat & Pedgaonkar 1973, Agarwal 1981, Williams & Rao 1981, Gupta et al. 1982).

Some of the fungi like *C. cladosporioides* are ubiquitous in nature. Shenoi and Ramalingam (1975) have recorded *C. cladosporioides* in air. The fungi responsible for head mold incidence are facultative saprophytes. After causing seed rot and seedling blight, the pathogens multiply in the field. The head mold pathogens were also isolated from the basal senescent leaves and plant debris and from soil. From these sources the fungal propagules get air-borne and bring about earhead infection.

It was observed that the anther lobes were retained in pearl millet cultivar IP 7042 up to the grain filling stage. Head mold incidence was common under humid weather conditions. This retention of anther lobes was for a longer period and humid weather probably aggravated the head mold problem. Incidentally, infections were reduced considerably on earheads whose anther lobes were detached artificially.

Stigma (Diehl 1950, Kozera 1968), nectar (Cauquill & Renney 1967), ovary wall, pericarp and integuments of the seed coat (Kozera 1968, Shinohara 1972) have been reported to act as entry points of infection on several instances (Neergaard 1977). During the current investigation, several air-borne fungi were observed to colonise the anther lobes initially. After getting a foothold on the anther lobes, the earhead infecting fungi started colonising the seeds of the earheads (Raveesha 1984).

It has been demonstrated that the pollen of pearl millet enhanced the germination of the spores of fungi like *Drechslera setariae* and *C. cladosporioides*. Pollen is reported to enhance the per cent germination, length of germ tube and rate of infection of many pathogens like *Helminthosporium sativum*, *Septoria nodorum* (Fokkema 1971), *Phoma betae* (Warren 1972), *Botrytis cinerea* (Borecka & Milikan 1973), *Claviceps purpurea* (Williams & Colotelo 1975), *Fusarium graminearum* (Naik & Busch 1979) and *Claviceps fusiformis* (Prakash 1983). Appaji Gowda and Ramalingam (1981) have demonstrated the influence of sorghum pollen on *D. turcica* conidia which led to an increase in the infection.

The first effect of earhead infection is the loss in grain production similar to sorghum head mold (Williams & Rao 1978). Wiehe (1953) noticed uneven grain production in pearl millet earheads infected by *Cladosporium herbarum*. Thus, seed abortion leads to grain loss. Sometimes, even if the seeds are produced, they will be very small, contributing to the reduction in weight.

Mathur et al. (1973) advocated the spraying of pearl millet plants with suitable fungicides to prevent the infection of seeds. Based

on the results of the present investigation, it is advised to spray the earheads with suitable fungicides at the time of grain filling stages to prevent losses. In breeding programme, plants which do not retain the anther lobes after anthesis should be selected and incorporated in reconstituting the high yielding cultivars.

Acknowledgements

The senior author is indebted to the University Grants Commission for financial assistance. Sincere thanks are due to Dr H S Prakash for his helpful discussion.

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Regd. No 16423/68

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Published by the Executive Secretary, Indian National Science Academy, Bahadur Shah Zafar Marg, New Delhi 110002 and Printed by him at The Central Electric Press, 80-D, Kamla Nagar, Delhi 110007